

**RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR  
SIMULTANEOUS DETERMINATION OF CODEINE  
PHOSPHATE, CHLORPHENIRAMINE MALEATE AND  
SODIUM BENZOATE IN COUGH SYRUP FORMULATION**

*Dissertation work submitted to  
The Tamilnadu Dr. M.G.R. Medical University, Chennai  
in partial fulfillment for the Award of Degree of*

**MASTER OF PHARMACY  
(PHARMACEUTICAL ANALYSIS)**

Submitted by

**N.DEEPIKA**

**Reg No. 26106421**

UNDER THE GUIDANCE OF

INSTITUTIONAL GUIDE

**Mr. P.R VIJAI ANAND, M. PHARM.,(Ph.D)**

Assistant Professor, Department of Analysis,

**RVS College of Pharmaceutical Sciences,**

**Sulur,Coimbatore.**

INDUSTRIAL GUIDE

**Dr. M. Usseni Reddy**

Manager, A R&D dep.

**VIVIMED LABS,**

**Hyderabad.**



**MAY 2012**

**DEPARTMENT OF PHARMACEUTICAL ANALYSIS**

**R.V.S. COLLEGE OF PHARMACEUTICAL SCIENCES**

**SULUR, COIMBATORE – 641402**

**TAMILNADU**

## **CERTIFICATE**

This is to certify that the dissertation work entitled “**RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE, CHLORPHENIRAMINE MALEATE AND SODIUM BENZOATE IN COUGH SYRUP FORMULATION**” is a bonafide work of **Ms. N.DEEPIKA(26106421)** carried out in VIVIMED LABS, HYDERABAD under the supervision of Dr. M. Usseni Reddy, and has completed to my fullest satisfaction for partial fulfilment of the award of degree of **Master of Pharmacy in Pharmaceutical Analysis**, RVS college of Pharmaceutical Sciences, Sulur, Coimbatore, which is affiliated to The Tamilnadu Dr.M.G.R Medical University, Chennai. It is to certify that the part or whole of the work has not been submitted either to this university or any other university. This work is original and confidential.

INSTITUTIONAL GUIDE

**Mr.P.R.VIJAI ANAND, M.Pharm.,(Ph.D)**

Date:

Assistant Professor,

Place:

Department of Pharmaceutical Analysis,

R.V.S College of Pharmaceutical Sciences

Sulur, Coimbatore-641402

## **CERTIFICATE**

This is to certify that the dissertation work entitled “**RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE, CHLORPHENIRAMINE MALEATE AND SODIUM BENZOATE IN COUGH SYRUP FORMULATION**” is a bonafide research work done by **Ms. N.DEEPIKA (26106421)** carried out in VIVIMED LABS, HYDERABAD, under the guidance of P.R.VIJAI ANAND, M.Pharm.,(Ph.D.), Assistant Professor, RVS College of pharmaceutical science, Sulur and Mr. Dr. M. Usseni Reddy, Manager, A R&D dep. VIVIMED LABS, HYDERABAD, for partial fulfilment of the requirement for the award of **Master of Pharmacy in Pharmaceutical Analysis**, R V S college of Pharmaceutical Sciences, Sulur, Coimbatore, which is affiliated to The Tamilnadu Dr.M.G.R Medical University, Chennai.

**Dr.R.VENKATANARAYANAN.M.Pharm., Ph.D.,**  
PRINCIPAL,

R.V.S College of Pharmaceutical Sciences  
Sulur, Coimbatore-641402.

Date:

Place:

## **CERTIFICATE**

This is to certify that the dissertation work entitled “**RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE, CHLORPHENIRAMINE MALEATE AND SODIUM BENZOATE IN COUGH SYRUP FORMULATION**” is a bonafide research work done by **Ms. N.DEEPIKA (26106421)** carried out in VIVIMED LABS, HYDERABAD, under the guidance of Mr. P.R VIJAI ANAND, M.Pharm.,(Ph.D.),Assistant Professor, RVS College of pharmaceutical science, Sulur and Mr. Dr. M. Usseni Reddy, for partial fulfilment of the requirement for the award of **Master of Pharmacy in Pharmaceutical Analysis**, RVS college of Pharmaceutical Sciences, Sulur, Coimbatore, which is affiliated to The Tamilnadu Dr.M.G.R Medical University, Chennai.

**Dr.W.D.SAMSOLOMON.M.Pharm., Ph.D.,**

Professor and Head,

Department of Pharmaceutical Analysis

R.V.S College of Pharmaceutical Sciences

Sulur, Coimbatore-641402.

Date:

Place:

## **CERTIFICATE**

This is to certify that the dissertation work entitled “**RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF CODEINE PHOSPHATE, CHLORPHENIRAMINE MALEATE AND SODIUM BENZOATE IN COUGH SYRUP FORMULATION**” is a bonafide research work done in VIVIMED LABS, HYDERABAD by **Ms. N.DEEPIKA(26106421)** in partial fulfilment of the requirement for the award of **Master of Pharmacy in Pharmaceutical Analysis**, RVS college of Pharmaceutical Sciences, Sullur, Coimbatore, under the supervision and guidance of **Mr.P.R.VIJAI ANAND. M.Pharm, (Ph.D.)**, Assistant Professor, Department of Pharmaceutical Analysis, RVS College of pharmaceutical science, Sullur and **Mr., Dr. M. Usseni Reddy**, Manager, A R&D dep. VIVIMED LABS, HYDERABAD.

**INTERNAL EXAMINER**

**Place:**

**Date:**

**EXTERNAL EXAMINER**

**Place:**

**Date:**

## **ACKNOWLEDGEMENT**

This dissertation would not have been possible without the guidance and the help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

First and foremost, my utmost gratitude to **Mr. P.R VIJAI ANAND** for his patience and steadfast encouragement to complete this study, whose sincerity and encouragement I will never forget. Thanks a lot for the advice you provided in paper publication for an International journal. Your assistance has been incredible to me during this process.

**Dr. R. Venkatanarayanan**, Professor and Principal, RVS College of Pharmaceutical Sciences, Sulur, Coimbatore had kind concern and consideration regarding my academic requirements.

**Dr. Sam Solomon, Mr. P. Kumarnallasivan, Mr.Sivakumar** for their inputs especially in the curriculum part of this study. They have shared valuable insights in the relevance of the study to basic education.

In my daily work I have been blessed with a friendly and cheerful group of my department members especially **Mr. BRAMAIH**, Associate analyst, AR&D, Vivimed Labs, Hyderabad who has taught me the validation procedures very effectively.

**P J NISHA**, for her unselfish and unfailing support as my dissertation adviser.

Last but not the least, my family and the one above all of us, the omnipresent God, for answering my prayers for giving me the strength, thank you so much Dear Lord.

**Date:**

**N. DEEPIKA**

**Place: Coimbatore**

**(Reg.No. 26106421)**

## **CONTENTS**

### **CHAPTER-1**

INTRODUCTION.....	1-39
-------------------	------

### **CHAPTER-2**

2.1 DRUG PROFILE.....	40-43
2.2 LITERATURE REVIEW.....	44-54
2.3 AIM & OBJECTIVE.....	55
2.4 PLAN OF WORK.....	56

### **CHAPTER-3**

#### EXPERIMENTAL DETAILS

3.1 MATERIALS AND METHODS.....	57-58
3.2 METHOD DEVELOPMENT.....	59-63
3.3 METHOD VALIDATION.....	64-75

### **CHAPTER 4**

#### 4.1 RESULTS & DISCUSSION

4.1.1 METHOD DEVELOPMENT.....	76-79
4.1.2 METHOD VALIDATION.....	80-111

SUMMARY SHEET.....	112
--------------------	-----

4.2 SUMMARY & CONCLUSION.....	113
-------------------------------	-----

### **CHAPTER 5**

BIBILOGRAPHY.....	114-118
-------------------	---------

## **LIST OF TABLES**

1. LIST OF INSTRUMENTAL METHODS.....	2
2. LIST OF COLUMN CHART SELECTION.....	14
3. LIST OF CHEMICALS USED.....	57
4. LIST OF INSTRUMENTS USED.....	58
5. OPTIMIZED CHROMATOGRAPHIC PARAMETERS.....	61
6. GRADIENT PROGRAMME OF ELUTION.....	61
7. VALIDATION PARAMETERS & REQUIREMENTS.....	65
8. ASSAY RESULTS OF CP,CPM & SB.....	79
9. SYSTEM SUITABILITY PARAMETERS.....	80
10. % RECOVERY STUDIES OF THE DRUGS.....	84
11. RESULTS FOR PRECISION.....	89
12. SPECIFICITY RESULTS OF CP,CPM & SB.....	93
13. LINEARITY RESULT FOR CODEINE PHOSPHATE.....	98
14. LINEARITY RESULT FOR CHLORPHENARAMINE MALEATE.....	99
15. LINEARITY RESULT FOR SODIUM BENZOATE.....	100
16. ROBUSTNESS RESULT FOR CHANGE IN FLOW RATE & P <sup>H</sup> .....	109
17. RUGUDNESS RESULTS OF ANALYSTS 1&2 ON DAYS 1&2.....	111
18. SUMMARY-RESULTS OF METHOD VALIDATION FOR CP, CPM & SB.....	112



## **LIST OF FIGURES**

1. SYSTEMATIC DIAGRAM-HPLC.....	12
2. LIST OF VALIDATION PARAMETERS.....	34
3. METHOD DEVELOPMENT TRAIL-1 CHROMATOGRAM.....	77
4. METHOD DEVELOPMENT TRAIL-2 CHROMATOGRAM.....	77
5. METHOD DEVELOPMENT TRAIL-3 CHROMATOGRAM.....	77
6. CHROMATOGRAM OF STANDARD SOLUTION OF CP, CPM & SB.....	78
7. CHROMATOGRAM OF TEST SOLUTION OF CP, CPM & SB.....	78
8. OVERLAY OF STANDARD & TEST SOLUTION OF CP, CPM & SB.....	79
9. SYSTEM SUITABILITY OF STANDARD SOLUTION OF CP, CPM & SB.....	81
10. CHROMATOGRAM OF ACCURACY-50% CP, CPM & SB.....	82
11. CHROMATOGRAM OF ACCURACY-100% CP, CPM & SB.....	83
12. CHROMATOGRAM OF ACCURACY-150% CP, CPM & SB.....	83
13. CHROMATOGRAM OF PRECISION-BLANK.....	85
14. CHROMATOGRAM OF PRECISION OF TEST SAMPLE-1.....	85
15. CHROMATOGRAM OF PRECISION OF TEST SAMPLE-2.....	86
16. CHROMATOGRAM OF PRECISION OF TEST SAMPLE-3.....	86
17. CHROMATOGRAM OF PRECISION OF TEST SAMPLE-4.....	87
18. CHROMATOGRAM OF PRECISION OF TEST SAMPLE-5.....	88
19. CHROMATOGRAM OF PRECISION OF TEST SAMPLE-6.....	88
20. CHROMATOGRAM OF METHOD PRECISION OF DRUGS.....	89
21. CHROMATOGRAM OF SPECIFICITY-BLANK SOLUTION.....	90
22. CHROMATOGRAM OF SPECIFICITY-STANDARD-1 SOLUTION.....	90
23. CHROMATOGRAM OF SPECIFICITY-STANDARD-2 SOLUTION.....	91
24. CHROMATOGRAM OF SPECIFICITY-STANDARD-3 SOLUTION.....	91
25. CHROMATOGRAM OF SPECIFICITY-STANDARD-4 SOLUTION.....	92
26. CHROMATOGRAM OF SPECIFICITY-STANDARD-5 SOLUTION.....	92
27. CHROMATOGRAM SHOWING OVERLAY OF BLANK & STNDRD SOL.....	93

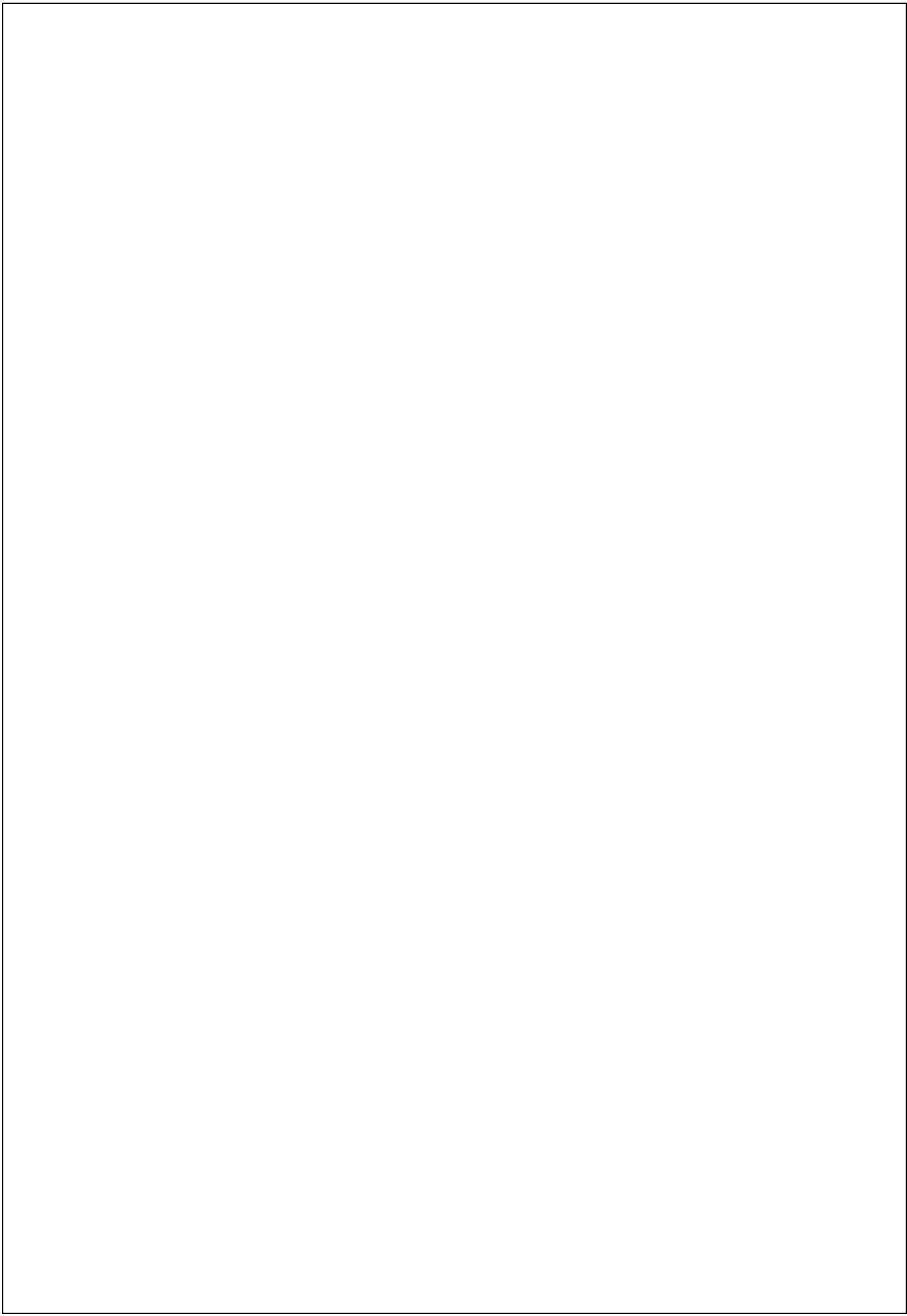
28. CHROMATOGRAM OF LINEARITY LEVEL-1(25%).....	94
29. CHROMATOGRAM OF LINEARITY LEVEL-2(50%).....	94
30. CHROMATOGRAM OF LINEARITY LEVEL-3(75%).....	95
31. CHROMATOGRAM OF LINEARITY LEVEL-4(100%).....	95
32. CHROMATOGRAM OF LINEARITY LEVEL-5(125%).....	96
33. CHROMATOGRAM OF LINEARITY LEVEL-6(150%).....	96
34. OVERLAY CHROMATOGRAM OF LINEARITY LEVELS OF CP, CPM & SB..	97
35. LINEARITY GRAPH OF CODEINE PHOSPHATE.....	98
36. LINEARITY GRAPH OF CHLORPHENARAMINE MALEATE.....	99
37. LINEARITY GRAPH OF SODIUM BENZOATE.....	100
38. LINEARITY GRAPH OF CP, CPM & SB.....	101
39. CHROMATOGRAM OF ROBUSTNESS-FLOW VARIATION(1.4ml/min)..	102-104
40. CHROMATOGRAM OF ROBUSTNESS-FLOW VARIATION(1.6ml/min)..	104-106
41. CHROMATOGRAM OF ROBUSTNESS-P <sup>H</sup> VARIATION(2.2).....	107
42. CHROMATOGRAM OF ROBUSTNESS-P <sup>H</sup> VARIATION(2.4).....	108
43. CHROMATOGRAM OF RUGUDNESS-DAY-1,ANALYST-1.....	110
44. CHROMATOGRAM OF RUGUDNESS-DAY-2,ANALYST-2.....	110

# **CERTIFICATES**

# ACKNOWLEDGEMENT

# INTRODUCTION

# LITERATURE REVIEW



# AIM & OBJECTIVE

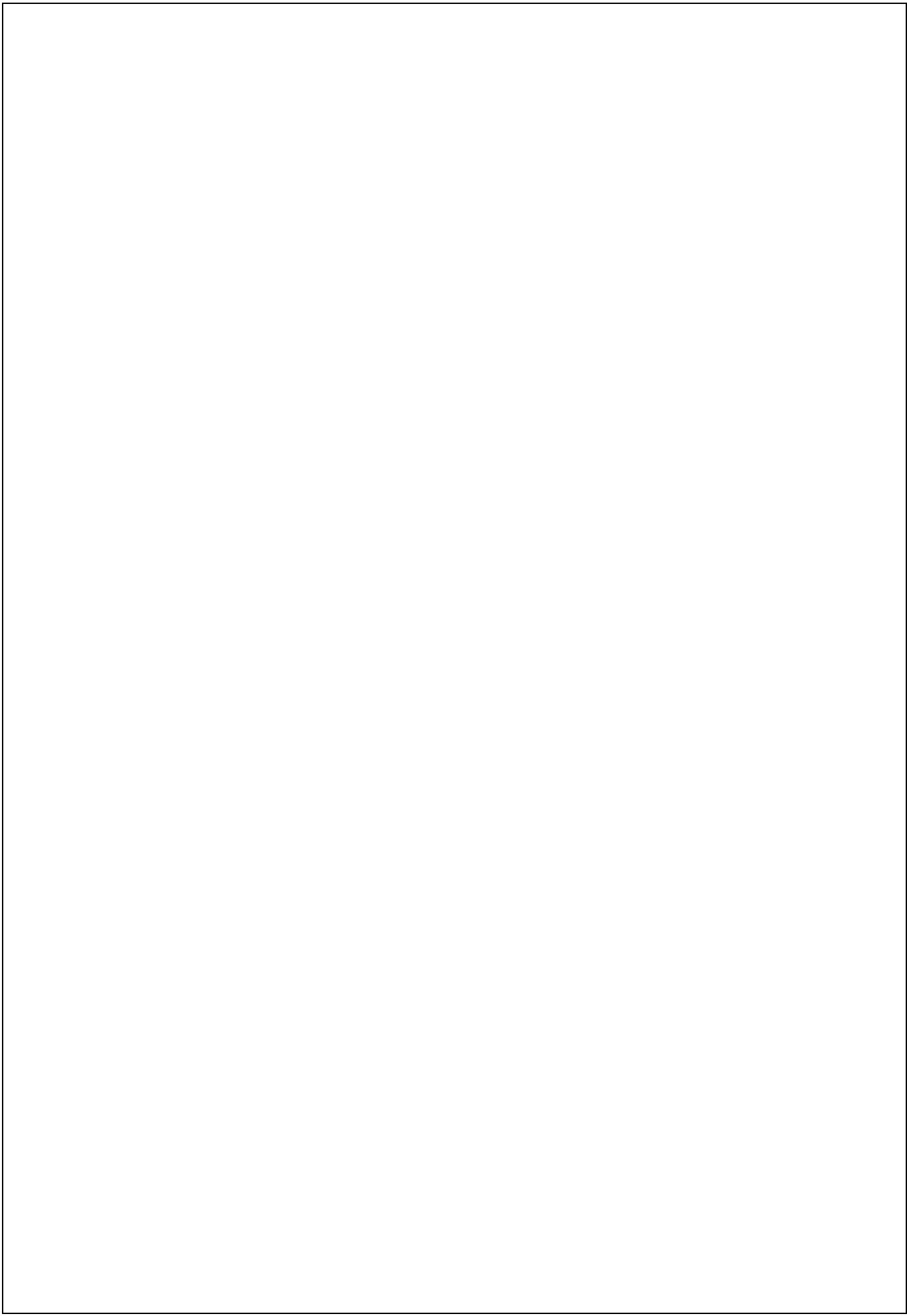


# PLAN OF WORK

# EXPERIMENTAL DETAILS

# VALIDATION PROCEDURES

# RESULTS & DISCUSSION



# SUMMARY & CONCLUSION

# BIBLIOGRAPHY

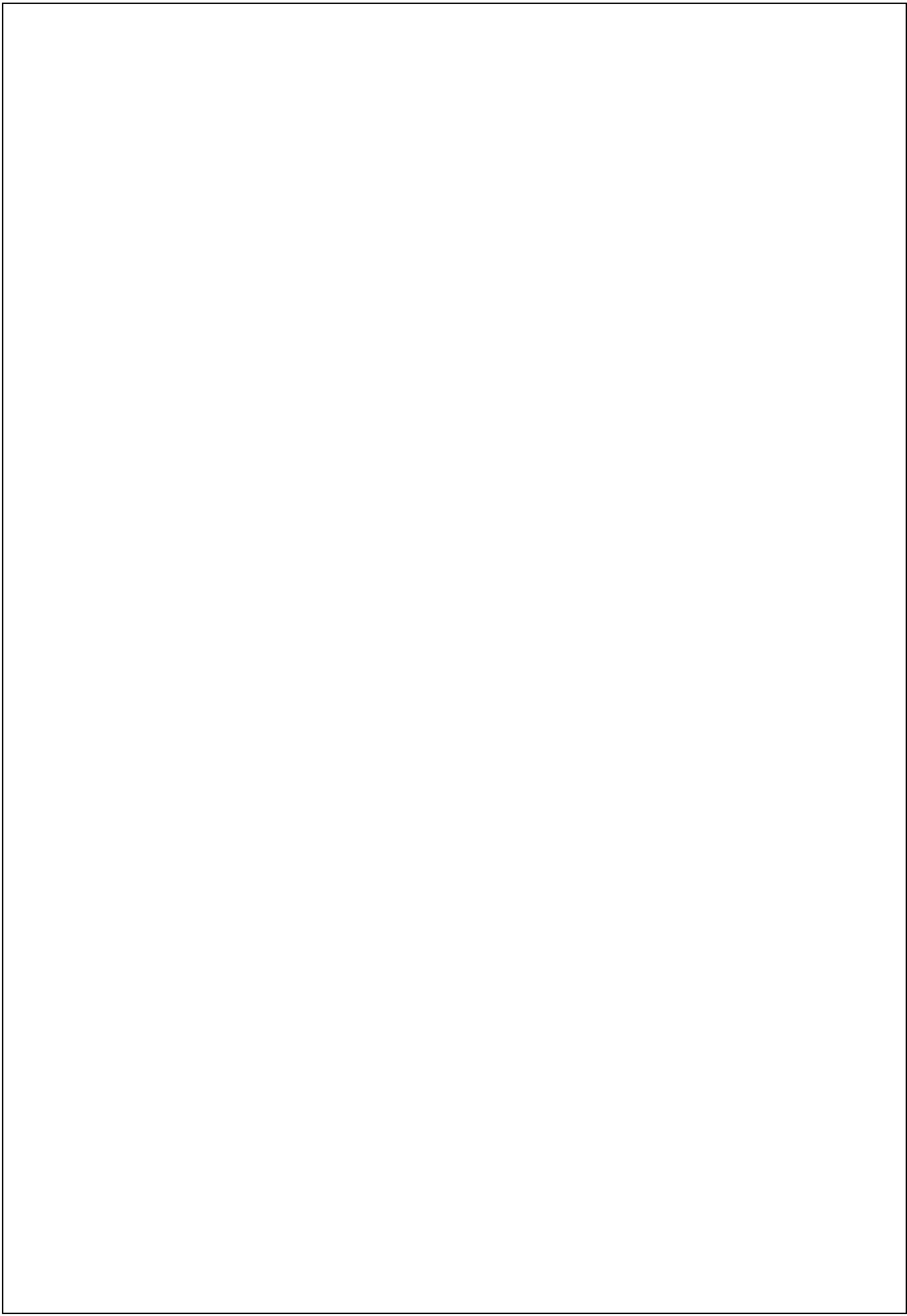
**Table no: 18 Table showing Summary of results of method validation for Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate**

S.No	Parameter	Requirement	Results			Acceptance criteria
			CP	CPM	SB	
1.	System suitability	RT	7.148	9.469	10.861	
2.		Tailing factor	1.1	1.0	1.1	NMT 2
3.		Resolution		11.7	8.6	NLT 2
4.		Plate count	16091.54	58142.60	63875.82	NLT 3000
5.		Assay value	99.2%	101.2%	99.8%	100 ± 2.0%
6.	Accuracy	% Recovery	98.7%	98.5%	99.1%	100 ± 2.0%
7.	Precision	%RSD	0.43	0.36	0.39	NMT 2%
8.	Specificity	No interference	Pass	Pass	Pass	No interference
8.	Linearity	Correlation coefficient	0.999991	0.999980	0.999943	NLT 0.999
9.	Range	Concentration	12.5-75 µg/ml	5-30 µg/ml	12.5-75 µg/ml	Nil
10.	Robustness	%RSD	0.41	0.38	0.39	NMT 1%
11.	Ruggedness	%RSD	0.55	0.34	0.64	NMT 1%



## **ABSTRACT**

A simple, specific, accurate and stability-indicating reversed phase high performance liquid chromatographic method was developed for the simultaneous determination of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate in cough syrup formulation. By Zodiac C-18, 3.5 $\mu$ , 150 $\times$ 4.6mm column eluted with Solvent-A: Phosphate buffer pH-2.3, Solvent-B: Acetonitrile by gradient elution pattern at a flow rate of 1.5 ml/min and a detection wavelength of 254 nm with injection volume of 50  $\mu$ l at Ambient(30°C) temperature afforded the best separation of these analytes. The retention times of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate were found to be 7.18 min, 9.469 min and 10.86 min respectively. The system precision of this method was evaluated by calculating the %RSD of the peak areas of six replicate injections of the standard solution, which were found to be 0.41%, 0.35% and 0.37%. Accuracy studies were performed where the % Recovery of three drugs was found to be 98.7%, 98.5% and 99.1% respectively. Linearity was established for Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate in the range of 12.5-75  $\mu$ g/ml, 5-30  $\mu$ g/ml and 12.5-75  $\mu$ g/ml respectively. Specificity of the current method was demonstrated by good separation of the three analytes from each other. Furthermore, excipients of the formulation did not interfere with the active ingredients of the drug product. Robustness of the current method was investigated by analyzing samples of the drug product using the same chromatographic conditions set forth in method development but with a small change in the following chromatographic parameters: Flow rate: 1.4 and 1.6 ml/min instead of 1.5 ml/min and pH of the buffer preparation in mobile phase: 2.2 and 2.4 instead of 2.3. The %RSD's were found to be within the limits. Ruggedness was demonstrated by analyzing three samples (assay) of syrup formulation by two analysts in the same laboratory on two different days. The %RSD values for the 12 samples are calculated to be 0.42%, 0.62%, and 0.49% for Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate respectively. The analytical procedure is validated as per ICH Q2B guidelines and shown to be accurate, precise and specific. This method is amenable to the routine analysis and can be successfully employed for simultaneous quantitative analysis of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate respectively in bulk drugs and formulations.



## 1. INTRODUCTION

### 1.1 PHARMACEUTICAL ANALYSIS:

Pharmaceutical Analysis is the branch of science which deals with identification of substances and determination of amount present in particular sample. Also pharmaceutical analysis deals with bulk materials, dosage forms and more recently, biological samples in support of bio-pharmaceutical and pharmacokinetic studies.

Analysis can be divided into areas called qualitative and quantitative analysis. Pharmaceutical products synthesized and identified using instrumental techniques.<sup>1</sup>

These methods are used extensively in the quality assurance of raw materials, in process quality assessment, stability of the drugs on storage and monitoring drugs concentrations in various body fluids or tissues.

The types of analysis can be distinguished in two ways:

**A. QUALITATIVE ANALYSIS:** To refer identity of product, i.e., it yields useful clues from which the molecular or atomic species, the structural features, or the functional groups in the sample can be identified.

**B. QUANTITATIVE ANALYSIS:** To refer the purity of the product, i.e., the results are in the form of numerical data corresponding to the concentration of analytes.

**TYPES OF ANALYTICAL METHODS**

The various methods of analysis<sup>3</sup> can be grouped into two categories. They are:

1. Chemical methods.
2. Instrumental methods.

**Chemical Methods:**

In these methods, volume and mass are used as means of detection.

1. Titrimetrical methods like acid-base, oxidation-reduction, non-aqueous, complexometric, precipitation titrations.
2. Gravimetric and thermo gravimetric methods.
3. Volumetric methods.

**Instrumental Methods:**

These methods are based on the measurement of specific and non-specific physical properties of a substance as given below:

Different Instrumental Methods Based On Principles

**Table No: 1 Table showing list of instrumental methods.**

S.No.	Principle	Instrument Method
1.	Emission of radiation.	X-ray emission spectrometry. Fluorescence spectrometry
2.	Absorption of radiation.	UV/Visible ,I.R Spectrophotometry, NMR Spectroscopy,ESR Spectroscopy, Atomic absorption spectrometry
3.	Mass to charge ratio.	Mass spectrometry
4.	Refraction of radiation.	Refractometry
5.	Scattering of radiation.	Nephelometry
6.	Rotation of radiation.	Polarimetry
7.	Electrical potential.	Potentiometry
8.	Electrical current.	Amperometry, Polargraphy

---

9.	Electrical resistance.	Conductometry
10.	Thermal properties.	Differential thermal analysis, Differential Scanning Calorimetry, Thermogravimetry
11.	Partition / Adsorption.	Chromatographic Techniques.

## 1.2 CHROMATOGRAPHY:

**Chromatography** (from Greek *chroma*, color and *graphein* to write) is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation. Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.

The history of chromatography begins during the mid-19th century. Chromatography, literally "color writing", was used and named in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll. New types of chromatography developed during the 1930s and 1940s made the technique useful for many types of separation process. Some related techniques were developed during the 19th century (and even before), but the first true chromatography.

**Chromatography terms**

- The **analyte** is the substance to be separated during chromatography.
- **Analytical chromatography** is used to determine the existence and possibly also the concentration of analyte(s) in a sample.
- A **bonded phase** is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing.
- A **chromatogram** is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture.

Plotted on the x-axis is the retention time and plotted on the y-axis a signal (for example obtained by a spectrophotometer, mass spectrometer or a variety of other detectors) corresponding to the response created by the analytes exiting the system. In the case of an optimal system the signal is proportional to the concentration of the specific analyte separated.

- A **chromatograph** is equipment that enables a sophisticated separation e.g. gas chromatographic or liquid chromatographic separation.
- **Chromatography** is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction.
- The **eluent** is the mobile phase leaving the column.
- An **eluotropic series** is a list of solvents ranked according to their eluting power.
- An **immobilized phase** is a stationary phase which is immobilized on the support particles, or on the inner wall of the column tubing.

- The **mobile phase** is the phase which moves in a definite direction. It may be a liquid (LC), a gas (GC), or a supercritical fluid (supercritical-fluid chromatography, SFC). The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. In the case of HPLC the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or polar solvents in reverse phase chromatography and the sample being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.
- **Preparative chromatography** is used to purify sufficient quantities of a substance for further use, rather than analysis.
- The **retention time** is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.
- The **sample** is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as waste.
- The **solute** refers to the sample components in partition chromatography.
- The **solvent** refers to any substance capable of solubilizing other substance, and especially the liquid mobile phase in LC.

- The **stationary phase** is the substance which is fixed in place for the chromatography procedure. Examples include the silica layer in thin layer chromatography.
- Techniques by chromatographic bed shape:
  - Column chromatography
  - Planar chromatography
    - Paper chromatography
    - Thin layer chromatography
- Displacement chromatography
- Techniques by physical state of mobile phase:
  - Gas chromatography
  - Liquid chromatography
- Affinity chromatography
  - Supercritical fluid chromatography
- Techniques by separation mechanism:
  - Ion exchange chromatography
  - Size exclusion chromatography
- Special techniques:
  - Reversed-phase chromatography
  - Two-dimensional chromatography
  - Simulated moving-bed chromatography
  - Pyrolysis gas chromatography
  - Fast protein liquid chromatography
  - Countercurrent chromatography
  - Chiral chromatography



**1.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:**

High Pressure Liquid Chromatography (HPLC) <sup>5</sup>, sometimes called High Performance liquid chromatography, is a separation that can be used for the analysis of organic molecules and ions. HPLC is based on mechanisms of adsorption, partition, ion exchange or size exclusion, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases.

The rate of distribution of drugs between stationary and mobile phase is controlled by diffusion process, if diffusion is minimized, a faster and effective separation can be achieved. The techniques of HPLC are so called because of its improved performance when compared to classical column chromatography. Advances in column technology, high pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation. <sup>6</sup>

HPLC is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed and the solvent(s) used. <sup>7</sup>

It offers following advantages:

- Speed (many analysis can be accomplished in 20 minutes or less)
- Greater sensitivity (various detectors can be employed)

- Reusable columns (expensive columns but can be used for many analysis)
- Ideal for the substances of low volatility.
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantitation.
- Precise and reproducible.
- Calculations are done by integrator itself.
- Suitable for preparative liquid chromatography on a much larger scale.

**TYPES OF HPLC <sup>8</sup>:****❖ Based on the mode of separation**

- Normal phase chromatography
- Reverse phase chromatography
- Partition chromatography
- Displacement chromatography

**❖ Based on principle of separation**

- Adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography
- Ion pair chromatography

**❖ Based on elution technique**

- Isocratic separation
- Gradient separation

**❖ Based on the scale of operation**

- Analytical HPLC

- Preparative HPLC

**Based on the mode of separation:****Normal phase chromatography:**

Also known as Normal phase HPLC (NP-HPLC) or Adsorption chromatography, separate analytes based on adsorption to a stationary surface chemistry and by polarity. It was one of the first kinds of HPLC that chemists developed. In this type stationary phase used is polar in nature and the mobile phase used is non-polar and non aqueous in nature. Depending on the nature of the analyte and stationary phase. If the affinity between the stationary phase and the analyte increases the retention time (RT) of the analyte also increases and vice versa. The interaction strength depends not only on the functional groups in the analyte molecule but also on steric factors. The effect of sterics on interaction strength allows this method to resolve (separate) structural isomers.

**Reverse phase chromatography**

In reverse phase technique, a non-polar stationary phase is used and the mobile phase is polar in nature. Hence polar components get eluted first and non-polar compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature, they are not retained for a longer time and eluted faster, columns used in the mode of chromatogram are ODS (Octadecyl silane) or C<sub>18</sub>, C<sub>8</sub>, C<sub>4</sub>, etc.

**Partition chromatography**

Partition chromatography was the first kind of chromatography that chemists developed. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid-liquid applications. Partition chromatography uses a retained solvent, on the surface or within the grains

or fibers of an "inert" solid supporting matrix as with paper chromatography; or takes advantage of some additional columbic and/or hydrogen donor interaction with the solid support. Molecules equilibrate (partition) between a liquid stationary phase and the eluent separate analytes based on the polar differences is known as Hydrophilic Interaction Chromatography (HILIC). Partition HPLC has been used historically on unbonded silica or alumina supports. Each works effectively for separating analytes by relative polar differences. However, HILIC has the advantage of separating acidic, basic and neutral solutes in a single chromatogram.

### **Displacement Chromatography**

The basic principle of displacement chromatography is a molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites and thus displace all molecules with lesser affinities. In elution mode, substances typically emerge from a column in narrow Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds and thereby be resolved there must be substantial differences in some interaction between the bio molecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings. Displacement chromatography has advantages<sup>4</sup> over elution chromatography in that components are resolved into consecutive zones of pure substances rather than "peaks".

**Based on principle of separation:****Adsorption chromatography:**

When a mixture of compounds (adsorbate) dissolved in the mobile phase (eluent) moves through a column of stationary phase (adsorbent) they travel according to their relative affinities. The compound which has more affinity towards stationary phase travels slower, if less affinity towards stationary phase travels faster.

**Ion exchange chromatography:**

It is the process by which a mixture of similar charged ions can be separated using ion exchange resin. There is a reversible exchange of ions between the ions present in the column. And those present in the ion exchange resin. For cations, cation exchange resin and for anions, an anion exchange resin is used.

- 1) Cation exchange eg: Sulfonated polystyrenes
- 2) Anion exchange resin eg: Carboxylic methacrylate

**Size exclusion chromatography:**

It is the process by which mixture of compounds with molecular sizes are separated by using gels. The gel used acts as molecular sieve. It can be separated by steric and diffusion effects of pores in the gels. The compound can separate according to the molecular sizes and the stationary phase is a porous matrix.

Eg: separation of proteins and polysaccharides.

**Chiral phase chromatography:**

In this type of chromatography, separation of optical isomers can be done by using chiral stationary phases i.e., levo and dextro form can be separated by using chiral stationary phases. Eg: chemically bonded silica gel.

**Ion pair chromatography:**

In this chromatography, a reverse phase column is converted temporarily into ion exchange column by using ion pairing agents like pentane or hexane.

**Based on the scale of operation:**

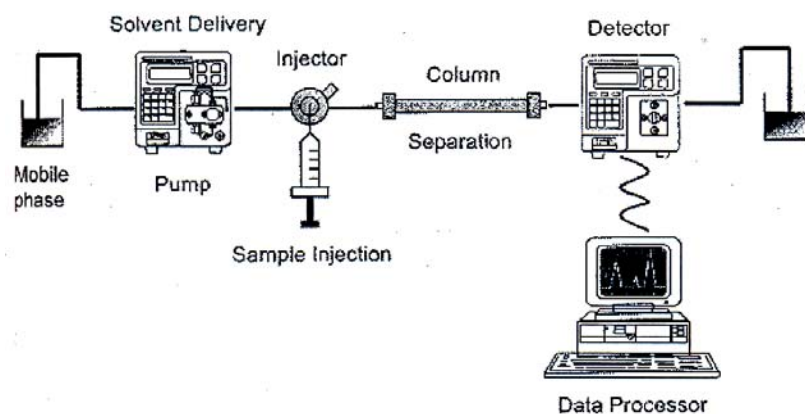
**Analytical HPLC:** It is used for analysis of samples. But recovery of samples is not done since the samples used are at very low level. Eg: microgram quantities.

**Preparative HPLC:** It is used for separation and collection of compound mixture, where the individual and pure compounds can be collected using fraction collector. And the collector samples are reused. Eg: separation of few grams of mixtures.

**INSTRUMENTATION OF HPLC<sup>9</sup>:**

The main components of HPLC are:

- Solvent Reservoir
- Pump
- Injection Port
- Column
- Detector
- Data Acquisition System



**Fig. No. 1: Figure showing Systematic diagram of HPLC.**

**Solvent Reservoir:**

Solvent Reservoirs are used to store Mobile-Phase. Scott Duran bottles are commonly used as solvent reservoirs. The solvent reservoir must be made of inert material such as glass and must be smooth so as to avoid growth of microorganisms on its walls. It can be transparent or can be amber colored. A graduated bottle gives a rough estimate of mobile-phase volume in the bottle. Solvent reservoirs are placed above HPLC system (at higher level) in a tray. They should never be kept directly above the system as any spillage of solvent on the system may damage electronic parts of HPLC.

**HPLC Pump:**

The HPLC pump is very important component of the system. The Pump delivers the constant flow of the Mobile Phase or phases so that the separation of the components of the mixture occurs in a reasonable time. There are two types of pumping systems Isocratic and Gradient. The gradient type pumps also classified into two types, those are:

- High pressure mixing. Eg: Mechanical and Piston pumps.
- Low pressure mixing. Eg: Pneumatic and Quaternary pumps.

**Injection Port <sup>10</sup>:**

The sample introduction device such as injector is to introduce the sample in a flow of mobile phase at high pressure. The valve injection through fixed or variable loop is a common way of introducing the sample. The Rheodyne valve is the mostly used devise.

**HPLC Column:**

The HPLC Column holds the stationary phase for separating the components of the sample. The columns are usually made up of SS-316 grade steel. Apart from

columns, the material of construction of tubing and fittings, plumbing and connections are also very critical. Apart from resistivity to corrosion, connections and plumbing should have very low dead volume.

**Table no: 2 list of column selection chart.**

<b>Column selection chart: molecular weight &gt; 2000 g/mol</b>						
	<b>Water soluble</b>					<b>Organic solvent soluble</b>
LC mode (packing)	RP bonded (C <sub>18</sub> , phenyl, C <sub>4</sub> )	aqueous gel filtration (SEC)	Ion exchange	hydrophobic interaction	affinity	size exclusion chromatography

### **Stationary phases:** <sup>11</sup>

There are many types of stationary phases employed in liquid chromatography including:

- (1) Silica, alumina or porous graphite is used in normal-phase chromatography where the separation is based on differences in adsorption and/or mass distribution.
- (2) Resins or polymers with acid or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase.
- (3) Porous silica or polymers, used in size-exclusion chromatography, where separation is based on differences between the volumes of the molecules, corresponding to steric exclusion.
- (4) A variety of chemically modified supports prepared from polymers, silica or porous graphite, used in reversed-phase liquid chromatography, where the separation is based principally on partition of the molecules between the mobile phase and the stationary phase.



- (5) Special chemically modified stationary phases, for example cellulose or amylase derivatives, proteins or peptides, cyclodextrins etc., for the separation of enantiomers (chiral chromatography).

**HPLC Detectors:**<sup>12</sup>

Detectors detect various compounds as they elute out from column. The detector gives response in terms of a millivolt (mv) signal that is then processed by the computer (integrator) to obtain you a chromatogram. Basically detector consists of a flow-cell through which the mobile phase and resolved sample moves optic shine through the detector cell and variation in optical properties are detected.

The Photo Diode Array Detector (PDA) is the most used detector in LC today. The PDA gives a three dimensional view of chromatogram (Intensity Vs Time) and Spectra (Intensity Vs Wavelength) simultaneously. It can be called as Spectro-chromatogram. The detailed analysis of the data reveals more information on the complexity of co elution and helps in identifying the merged peaks and gives information on peak purity.

**Various types of HPLC Detectors:**

There are several types of detectors available in the market. Those are

- UV-VIS Detector
- Photo-Diode Array Detector (PDA)
- Fluorescence Detector
- Conductometric and colorimetric detector
- Mass detector
- Evaporative Light Scattering detector (ELSD)

Among these detectors Photo Diode Array Detector (PDA) is the most widely used detector. The PDA gives a three dimensional view of chromatogram (Intensity

Vs Time) and Spectra (Intensity Vs Wavelength) simultaneously. It can be called as Spectro-chromatogram. The detailed analysis of the data reveals more information on the complexity of co elution and helps in identifying the merged peaks and gives information on peak purity.

**Ideal Characteristics of a Detector:**

- Either is equally sensitive to all eluted peaks.
- The ideal detector give the response (area) proportional to the amount injected, irrespective of the size of sample.
- Cheap, reliable and easy to use.
- Should not be affected by change in temperature or mobile phase composition.
- It should be able to monitor small amounts of compound.

**i. Bulk property detector:**

These provide a differential measurement of bulk property possessed by both the solute and mobile phase Ex: Refractive index. It is not suitable for gradient elution and is less sensitive.

**ii. Solute property detector:**

This measures physical or chemical property that is specific to the solute only. Ex: U.V detector, conductivity detector. These can be used for gradient elution.

The detection of the separated compounds in the elute from the column is based up on the bulk properties of the elute are the solute property of the individual components generally, a detector is selected that will respond to a particular property of the substances being separated, and ideally it should be sensitive to at least  $10^{-8}$ g ml<sup>-1</sup> and give a linear response over a wide concentration range.

**Data collection devices:**

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The data storage capacity of these devices is usually limited.

Modern data stations are computer based and have a large storage capacity to collect process and store data for possible subsequent reprocessing. Analytical reports can often be customized to the needs of the analyst.

Integration of peak areas and the setting of threshold levels are not normally problematic in an assay since the peak of the substance to be analysed should be free of interference. However, in a test for impurities, the selection of the peak area integrator parameters becomes very important, particularly when baseline separations are not always attainable. If baseline separations cannot be obtained, valley-to-valley integration should be employed.

HPLC allows limits to be set for individual impurities and for the sum of impurities, but there is a level at which peaks should not be integrated. This "disregard level" is set in relation to the area of the peak in the chromatogram of the prescribed reference solution and is usually equivalent to 0.05% of the substance being examined.

**CHROMATOGRAPHY PARAMETERS<sup>13</sup>:****System suitability:**

The system suitability test represents an integral part of the method and is used to ensure the adequate performance of the chosen chromatographic system.

Efficiency, capacity factor resolution factor, and symmetry factor are the parameters that are normally used in assessing the column performance. Factors that can affect chromatographic behavior include mobile phase composition, temperature,

ionic strength, apparent pH, flow rate and column length and stationary phase characteristics such as porosity, particle size and type, and specific surface area.

**Efficiency (N) <sup>14</sup> :**

The efficiency of a chromatographic column is defined in terms of the number of theoretical plates (N) and can be calculated using the following formula:

$$N = 5.54 \frac{t_R^2}{W_h^2}$$

Where,  $t_R$  = retention time or the baseline distance between the point of injection and the perpendicular dropped from the maximum of the peak of interest.

$W_h$  = the width of the peak of interest determined at half peak height, measured in the same units as  $t_R$ .

N = The number of theoretical plates per meters.

The column plate number increases with several factors:

1. Well-packed columns (column “quality”)
2. Longer columns
3. Lower flow rates (but not too low)
4. Smaller column-packing particles
5. Lower mobile-phase viscosity and higher temperature
6. Smaller sample molecules.

**Capacity factor (mass distribution ratio,  $D_m$ ):**

This factor determines the retention of a solute and can be calculated from the chromatogram using the following formula:

$$D_m = \frac{(t_R - t_M)}{t_M}$$

Where,  $t_R$  = retention time of the solute

$t_M$  = retention time of an unretained component

A low  $D_m$  value indicates that the peak elutes close to the solvent front, which may compromise selectivity. A minimum  $D_m$  value of 1 is recommended for the peak of interest.

The retention time of the test substance can be varied, if necessary, by changing the relative proportion or composition of solvents in the mobile phase. Generally, an increase in the proportion of a more polar solvent will lead to a shorter retention time on a normal-phase column and a longer retention time on a reversed-phase column.

**Resolution factor ( $R_s$ ):**

It is measure of the extent of separation of two compounds and the baseline separation is achieved.

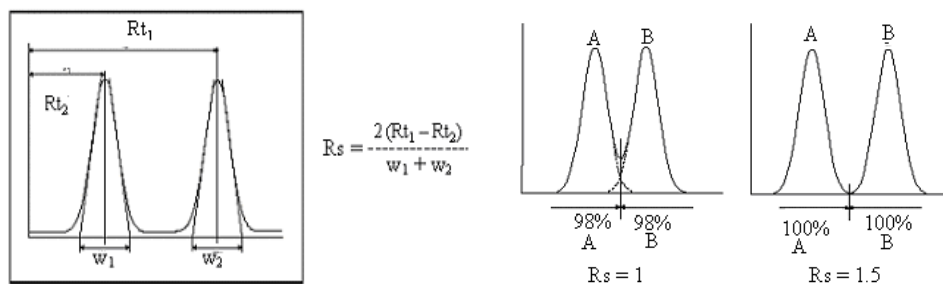
The resolution between two peaks of similar height in a chromatogram can be calculated using the following formula:

$$R_s = \frac{1.18(t_{R2} - t_{R1})}{(W_{b1} + W_{b2})}$$

Where,  $t_{R1}$  and  $t_{R2}$  = retention times or baseline distances between the point of injection and the perpendicular dropped from the maximum of each of the two peaks.

$W_{b1}$  and  $W_{b2}$  = the respective peak widths determined at half peak height, measured in the same units as  $t_{R1}$  and  $t_{R2}$ .

The value of  $R_s$  for a baseline separation between peaks of similar height should be at least two.

**Relative retention:**

The relative retention ( $r$ ) is calculated as an estimate using the following formula:

$$r = \frac{t_{R2} - t_M}{t_{R1} - t_M}$$

Where,  $t_{R2}$  = retention time of the peak of interest

$t_{R1}$  = retention time of the reference peak

$t_M$  = retention time of an unretained component

**Retention time ( $R_t$ ):**

Retention time is the difference in time between the points of injection and eluted from a column. Retention time is measured in minutes or seconds. Retention time is also proportional to the distance moved on a chart paper, which can be measured in cm or mm.

**Retention volume ( $V_r$ ):**

Retention volume is the volume of mobile required to elute 50% of the component from the column. It is the product of retention time and flow rate.

$$\text{Retention volume (Vr)} = \text{Retention time (Rt)} \times \text{flow rate}$$

**Column Efficiency (N):**

It is called as the number of theoretical plates. It measures the band spreading of a peak. When band spread is smaller, the number of theoretical plates is higher. It indicates a good column and system performance.

$$N = 16 (RT / W)^2$$

**HETP (High Equivalent Theoretical Plates):**

A theoretical plate can be of any height, which decides the efficiency of separation. If HETP is less the column is more efficient. If HETP is more, the column is less efficient. The height equivalent to a theoretical plate (HETP) is given by-

$$\text{HETP} = \text{Length of column (L)} / N$$

Where N = plates per meter

RT = retention time of the components.

W = width of the base of the component peak using tangent method.

L = column length in meters

**Symmetry factor ( $A_s$ ):**

The symmetry factor for a peak can be calculated using the following formula:

$$A_s = \frac{W_x}{2d}$$

Where,  $W_x$  = width at 5% of peak height measured from the baseline.

$d$  = baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of the peak height, measured in the same units as  $W_x$ .

Values of  $A_s$  which are greater than 2 may lead to incorrect integration, resulting in erroneous quantitation. The main factors that influence peak symmetry depend upon retention, solvent effects, incompatibility of the solute with the mobile phase or development of an excessive void at the inlet of the column. In reversed-phase chromatography, adsorption phenomena due to the presence of residual silanol groups in the stationary phase may lead to tailing (poor peak symmetry).

**Tailing Factor (T):**

The Tailing Factor T, a measure of peak symmetry is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. In some cases, values less than 1 may be observed. As peak asymmetry increases integration and hence precision becomes less reliable.

$$T = W_{0.05} / 2f$$

Where,  $W_{0.05}$  = width of peak at 5% height

$f$  = Distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline. Limit:  $\leq 2$ .

**1.4 METHOD DEVELOPMENT:**

The need to save method development time and improve accuracy is forcing today's analytical chemists to look for better, faster ways to develop stability indicating methods. Starting with HPLC columns that offer excellent reproducibility, column lifetime and sensitivity this step-by-step protocol can save the method development chemist time and money required to establish new method. This approach is consistent with developing process.

**PROCEDURE (PROTOCOL) FOR METHOD DEVELOPMENT****Literature Survey:**

Conduct literature survey and collect information available from the following references

- Chemical abstracts
- Analytical abstracts
- Journals
- National library of medicines etc,



And collect the following literature from survey

**Solubility profile:** Solubility of drug in different solvents at different pH conditions which is useful while selecting the diluents for standard solution and extraction solvents for test solution.

**Analytical profile:** Physico-chemical and spectroscopic properties, impurity and degradation profile of drug substance. Spectral profile is useful in the selection of detector wavelength for analysis, where as degradation profile helps to develop the method for separation of all possible impurities and degradants from API.

**Stability profile:** Stability of the drug with storage conditions. This helps to adopt suitable and adequate precautions while handling drug substances and its solutions.

The various parameters that include to be optimized during method development are:

- Selection of Mode of Separation
- Selection and Optimization of Mobile Phase
- Selection of Detector Wavelength
- Selection of Column
- Selection of Solvent Delivery Systems
- Selection of Flow Rate
- Selection of Column Temperature
- Selection of Diluent and Extraction Procedure
- Selection of test concentration and injection volume
- Establishment of Stability of the Solutions
- Establishment of System Suitability

**Selection of Mode of Separation:**

In Reverse Phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most

preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix.

**Selection and Optimization of Mobile Phase:**

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all individual impurities and degradants from analyte peak. The selection of mobile phase is done always in combination with selection of column (stationary phase). The following are the parameters, which shall be taken into consideration during the selection and optimization of mobile phase.

- Buffer, if any and its strength
- pH of buffer or pH of mobile phase
- Mobile phase composition

**Buffer if any and its strength:**

Buffer and its strength play an important role in deciding the peak symmetries and separation. Various types of buffers can be employed for achieving the required separation. The following are some of the most commonly used ones.

- Phosphate buffers :  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{Na}_2\text{HPO}_4$ ,  $\text{H}_3\text{PO}_4$
- Acetate buffers : Ammonium acetate, Sodium acetate
- Amine buffers : Triethyl amine/ Diethyl amine
- Buffers with various ion pair reagents like Tetra Butyl ammonium hydrogen sulphate
- Butane sulphonic acid, Hexane sulphonic acid, Heptanes sulphonic acid etc,

It is important to use the buffers with suitable strength to cope up for the injection load on the column otherwise peak tailing may arise due to changes in ionic form during chromatography. The retention times also depends on the molar strength of the buffer since molar strength is inversely proportional to retention time.

Ideally, the strength of the buffer shall be adopted in-between 0.05M to 0.2M. The selection of the buffer and its strength is done always in combination with selection of organic phase composition in mobile phase. The strength of the buffer can be altered if necessary to achieve the required separation. But it has to be ensured that the higher strength of the buffer shall not result in precipitation/turbidity either in mobile phase or in standard and test solution while allowed standing in bench top or in refrigerator. Experiments shall be conducted using different buffers having different strength to obtain the required separation.

The buffer having a particular strength, which gives separation of all individual impurities from API peak, shall be selected. Then strength of the buffer can be varied by about 10 to 20 % from the selected buffer strength and the effect of variation shall be studied. After reviewing the results of variation, the buffer and its strength shall be selected, this is rugged for at least 2% variation in strength.

#### **pH of the buffer or pH of the mobile phase:**

pH plays an important role in achieving the chromatographic separation as it control the elution properties by controlling the ionization characteristics. Depending on the pKa, drug molecule changes retention.

E.g.: Acids show an increase in retention as pH decreases, while bases show decrease in retention time.

Experiments shall be conducted using buffers having different pH to obtain the required separation. It is important the pH of the mobile phase in the range of 2.0 to 8.0 as most of the columns doesn't withstand to a pH outside this range. This is due to fact that the siloxane linkages are cleaved below pH 2.0, while at pH values above 8.0 silica may dissolve.

If a pH outside this range is found necessary, packing materials, which can withstand these ranges shall be chosen. pH of the buffer, which gives separation of all individual impurities from each other and from API, shall be selected. Then pH is varied by  $\pm 0.2$  from the selected pH and effect of variation shall be studied. After reviewing the results, a pH is selected which is rugged at least for  $\pm 0.2$  of the selected pH.

**Mobile phase composition:**

In reverse phase chromatography, the separation is mainly controlled by the hydrophobic interactions between drug molecule and the alkyl chains on the columns packing material. Most chromatographic separations can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are methanol and Acetonitrile. Tetra hydro furan is also used but to a lesser extent.

Experiments shall be conducted with mobile phase having buffers with different pH and organic phases to check for the best separation between the impurities. A drug solution having all possible known impurities can be used for checking the extent of separations with different mobile phase ratios. Alternatively solution of stressed drug substance can be used to check for the separation of impurities. A mobile phase composition which gives separation of all possible impurities and degradants from API Peak and which is rugged at least for  $\pm 0.5$  in both aqueous and organic phase shall be selected.

In reverse phase, methods are developed using buffers at the specified mobile phase pH. Optimum buffering capacity occurs at a pH equal to the pKa of the buffers. Also it shows little effect on retention time. Potassium salts are more soluble than

sodium salts. Diammonium hydrogen orthophosphate is used as phosphate buffer pH 2.3 and Acetonitrile.

**Selection of Detector Wavelength:**

Selection of detector wavelength is a critical step in finalization of the analytical method. To determine the exact wavelength standard API is injected into chromatographic system with Photo Diode Array detector and the wave length, which gives higher response for the compound will be selected. An ideal wavelength is one that gives good response for the drugs to be detected. In order to ascertain the optimum wavelength ( $\lambda_{\text{max}}$ ) of the species formed. A 10 $\mu\text{g/ml}$  of sample solution was prepared and scanned under UV-visible spectrophotometer in the range of 200 to 400nm against diluent as blank. The wave length selected based on the maximum absorption occurred.

**Selection of Column:**

Column plays the most important role in achieving the chromatographic separation. The following parameters should be considered while selecting a column.

- Length and diameter of the column
- Packing material
- Size and shape of particles
- Pore size, surface area and end capping
- Percentage of carbon loading

Columns with silica as a packing material is used widely in **Normal phase chromatography**, where the eluent (mobile phase) is non-polar consisting of various organic solvents and the stationary phase is polar. The silanol groups on the surface of the silica give it a polar character.

In **Reverse phase chromatography** a wide variety of columns is available covering a wide range of polarity by cross linking the silanol groups with alkyl chains like C<sub>6</sub>, C<sub>8</sub>, C<sub>18</sub> and Nitrile groups (-CN), Phenyl groups (-C<sub>6</sub>H<sub>5</sub>) and amino groups (-NH<sub>2</sub>).

### **ORDER OF THE SILICA BASED COLUMNS**

I-----Non Polar-----Moderately Polar-----Polar-----I

C<sub>18</sub> < C<sub>8</sub> < C<sub>6</sub> < Phenyl < Amino < Cyano < Silica

Experiments are conducted using different columns with different mobile phase to achieve best separation. A column which separates all the impurities and degradants from API peak and which is rugged with mobile phase variation is selected.

### **Selection of Solvent Delivery Systems:**

Chromatographic separations with single eluent i.e., all the constituents of mobile phase is mixed and pumped as single eluent is called Isocratic Elution and is always preferable. However Gradient Elution is a powerful tool in achieving separation between closely eluting compounds having different polarities. The importance of Gradient Elution is that the polarity and Ionic strength of the mobile phase can be changed during the run. Gradient elution is of two types

1. Low pressure Gradient and
2. High pressure Gradient Elution

Low pressure Gradient is one in which mobile phases are mixed at pre determined ratios and in High pressure Gradient mobile phase are pumped at different flow rates to achieve the required composition and mixed in mixing chamber and then introduced to the column. Low pressure Gradient is opted when NMT 80% of organic phase has to be pumped. High pressure Gradient is opted when more than 80% of

organic phase has to be pumped. While optimizing the gradient elution it is important to monitor the following-

- The graph is to be monitored so as to ensure that the overall system pressure will not cross 300 bars at any point during the run.
- Flow rate is to be physically cross checked by collecting the output from the detector during the run at different time intervals. This avoids pumping problems which may arise due to higher organic phase compositions.

**Selection of Flow Rate:**

Flow rate is selected based on the following factors

- Retention time
- Column composition
- Separation impurities
- Peak symmetry

Preferably flow rate shall not be more than 2.5 mL/Min. A flow rate that gives least retention times, good peak symmetries, least back pressure and better separation of impurities from API peak shall be selected.

**Selection of Column Temperature:**

Ambient temperature is always preferred as column temperature. However if the peak symmetry could not be achieved then the column temperature can be varied between 30° to 80°C. If a column temperature above 80°C is found necessary, packing material which can withstand to that temperature shall be chosen. The increase in column temperature generally will result in reduction in peak asymmetry and peak retentions.

For developing a method, the type of column being used and functionality, structure, pKa and reactivity must be considered. Optimize the temperature and adjust the

mobile phase compositions to achieve best separation. Usually the silica column temperatures stable up to 60°C, optimized flow rate achieve the shortest analysis and adequate resolution and efficiency. In Isocratic method performs the analysis at 50°C. Reduce the amount of organic modifier in the mobile phase is necessary to produce the good separation. Some column pre heaters are used to preheat the mobile phase to avoid broad broadening. Also use peltier chip for precise temperature and controls the column effluent. Silica based stationary phases stable at up to 60°C some instances up to 90°C. the higher temperature will leads the shorter column life time. At elevated temperature the solute transfer from mobile phase to the stationary phase is more efficient.

#### **Selection of Diluent and Extraction Procedure:**

Diluent for test preparation is selected initially based on solubility of the drug substance. It is selected in such a way that the drug substance is soluble in which the extraction is complete, due to which there won't be any interference and in which peak symmetry and resolution between impurities and API Peak is found satisfactory. General methods followed for extraction are Sonication, Rotatory shaking or both. In some cases where API is not extracted by above methods then heating is adopted if substance is stable and it should not precipitate upon cooling to room temperature.

Experiments are conducted to optimize the extraction of API in the presence of excipients at different test concentrations using the diluents chosen based on solubility at different time intervals of sonication time or rotary shaking or both and select the test concentrations at which the extraction is most efficient.

The solubility of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate was determined in a variety of solvents. As per Indian Pharmacopoeial standards solubility test for these drugs are carried out in different polar and non-polar



solvents. From the solubility studies water was chosen as solvent for the present drugs.

**Selection of test concentration and injection volume:**

The test concentration is generally chosen based upon the response of API peak at the selected detector wavelength. However test concentration is finalized after it is proved that API is completely extractable at the selected test concentration. Generally an injection volume of 10 to 20 $\mu$ L is recommended for estimation of API. However if the extractions are found to be difficult then the test concentration can be kept low and the injection volume can be increased up to 50 $\mu$ L. But it is to be ensured that at the selected volume the column is not overloaded.

**Establishment of Stability of the Solutions:**

The assessment of stability of the solution is carried out by keeping the test solution at Room Temperature and at 2° to 8°C and this test solution is injected along with the freshly prepared standard and %RSD of the standard solution and test solution is calculated. An acceptance criterion here is that the %RSD should not be NMT 2%.

**Establishment of System Suitability:**

System suitability parameter has to be selected based on the Tailing factor, Plate count, Resolution, and RSD. In general resolution factor for the closely eluting compounds is selected as a system suitability requirement. If the separation of impurities from each other and from API peak is found to be satisfactory, there is no need to keep a resolution factor as system suitability parameter. In such cases only standard reproducibility and symmetry of standard peak can be adopted as a system suitability requirement.

## 1.5 METHOD VALIDATION <sup>15</sup>:

Validation by definition is an act of proving that any procedures, process, equipment, materials, activity or system performs as expected under a given set of conditions. Basically validation is proving that the performance is as intended when extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by some different persons, in same or different laboratories using different reagents, different equipments etc.

According to **U. S. FDA** defines the term “Validation is a process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce meeting, its predetermined specifications and quality attributes”.

According to **USP**, “Validation of an analytical method is the process by which it is established by laboratory studies that the performance characteristic of the method meets the requirements for the intended in analytical applications”.

### **Purpose of Validation:**

1. Enable the scientists to communicate scientifically and effectively on technical matter.
2. Setting the standards of evaluation procedures for checking compliance and taking remedial action.
3. Economic: Reduction in cost associated with process sampling and testing.
4. As quality of the product cannot always be assured by routine quality control because of testing of statistically insignificant number of samples.

### **Types of Analytical Procedures to be validated:**

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:-

- Identification tests;
- Quantitative tests for impurities' content;
- Limit tests for the control of impurities;
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Although there are many other analytical procedures, such as dissolution testing for drug products or particle size determination for drug substance, these have not been addressed in the initial text on validation of analytical procedures. Validation of these additional analytical procedures are equally important to those listed herein and may be addressed in subsequent documents.

A brief description of the types of tests considered in this document is provided below.

1. Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behavior, chemical reactivity, etc) to that of a reference standard.
2. Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures (e.g., dissolution).

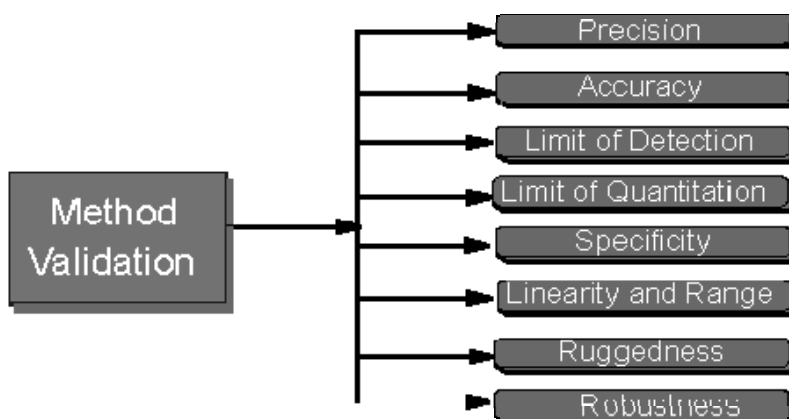
Typical validation characteristics which should be considered are listed below:

- Accuracy
- Precision
- Repeatability
- Intermediate Precision
- Specificity
- Detection Limit
- Quantitation Limit
- Linearity
- Range

Furthermore revalidation may be necessary in the following circumstances:

- Changes in the synthesis of the drug substance
- Changes in the composition of the finished product
- Changes in the analytical procedure

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.



**Fig. No. 2: Figure showing Validation parameters.**

**ACCURACY:**

It is the closeness of agreement between the actual value of the drug and the measured value. Spike and recovery Studies are performed to measure accuracy, a known sample is added to the excipients and the actual drug value is compared to the value found by the assay. Accuracy is expressed as the bias or the % error between the observed value and the true value (assay value/actual value x 100%).

**PRECISION:**

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

**REPEATABILITY:**

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

**INTERMEDIATE PRECISION:**

Intermediate precision expresses within-laboratories variations; different days, different analysts, different equipment, etc.

**REPRODUCIBILITY:**

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

It is expressed as the coefficient of variation (% CV). CV is the standard deviation of the assay values divided by the concentration of the analyte. Several types of precision can be measured: intra-assay precision (repeatability) is the % CV of multiple determinations of a single sample in a single test run; inter-assay precision (also called intermediate precision) measures the % CV for multiple determinations of a single sample, controls and reagents analyzed in several assay runs in the same laboratory.

**LINEARITY:**

A linearity study verifies that the sample solutions are in concentrations range where analyte response is generally performed by preparing standard solutions at five concentration levels from 50 to 150% of the target analyte concentrations five levels are required to allow detection of curvature in the plotted data.

Validation over a wide range provides confidence that the routine range provides confidence that the routine standard levels are well removed from non linear response concentrations that the method covers a wide enough range to incorporate the limits of content uniformity testing.

Acceptance of linear data is often judged by examining the correlation coefficient  $>0.999$  is generally considered as evidence of acceptable fit of the data. For the evaluation of linearity five different concentrations of standard solutions were prepared. The concentration ranges that prepared were between 25 to 125  $\mu\text{g/ml}$  for. A graph is plotted to “area” versus amount found”. The co-relation coefficient was found to be 0.999. It is the ability of an assay to obtain test results, which are directly proportional to the concentration of an analyte in the sample. The determination of this parameter will identify the range of the analytical assay. It can

be measured as slope of the regression line and its variance or as the coefficient of determination and correlation coefficient (R).

**RANGE:**

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results (e.g., percentage, parts per million) obtained by the analytical method.

**SPECIFICITY:**

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implications:

Identification: to ensure the identity of an analyte.

Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency): To provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

**LIMIT OF DETECTION (LOD):**

It is the lowest amount of the analyte in a sample that can be detected but not necessarily be quantitated as an exact concentration or amount. The detection limit of an individual analytical procedure is the lowest amount of an analyte in the sample

that can be detected but not necessarily quantitated as an exact value. The Limit of LOD value is 3.

For determining LOD initially standard deviation and slope of calibration curve was calculated.

$$\text{LOD} = 3.3 \text{ SD}/S_1$$

$$\text{LOD} = S/N$$

Where,  $S_1$  = slope of calibration curve

SD = standard deviation of peak response

S = signal obtained from the LOD solution.

N = average base line noise.

#### **LIMIT OF QUANTITATION (LOQ):**

It is the lowest amount of an analyte that can be measured quantitatively in a sample with acceptable accuracy and precision. The LOQ is a parameter for tests measuring impurities in a drug product.

The quantification limit of an individual procedure is the lowest amount of analyte in the sample that can be quantitatively determined with suitable precision and accuracy. The limit of LOQ value is 10.

For determining LOQ initially standard deviation and slope of calibration curve was calculated.

$$\text{LOQ} = 10 (\text{SD}/S)$$

Or

$$\text{LOQ} = S/N$$



**ROBUSTNESS:**

It is the capacity of an assay to remain unaffected by deliberate changes to various parameters of the method and gives an indication of its reliability during normal assay conditions. The variations could be in room or incubator temperature or humidity, variations in incubation times, minor variations in pH of a reagent, etc.

**SYSTEM SUITABILITY:**

Tailing factor for the peaks due to Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate Standard solution should not be more than 1.5. Theoretical plates for the peaks in Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate Standard solution should not be less than 2500.

**ASSAY:**

AT	WS	DT	P	Wt/ml	
-----	x	-----	x	-----	x ----- X 100
AS		DS	WT	100	Label Claim

Where:

AT = Peak Area of Standard solution.

AS = Peak Area of test solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

>The limit of assay is in between the 98% - 102%.

## 2.1 DRUG PROFILE

### COUGH SYRUP FORMULATION

**Description:** It is orange red coloured flavor based syrup.

**Category:** Antitussive.

**Composition:**

Each 5ml syrup contains:

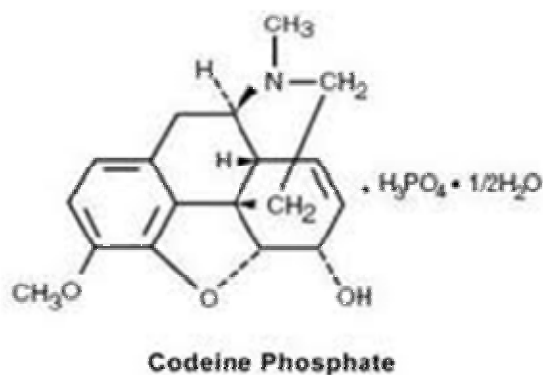
- (a) Codeine phosphate -----10mg
- (b) Chlorpheniramine Maleate ----- 4mg
- (c) Sodium Benzoate ----- 10mg

The general formulations known as cough medicines contain several active ingredients. They are usually a combination of an antitussive, antihistaminic drug.

The pure drugs are obtained from in-house for analysis.

### 2.1.1 CODEINE PHOSPHATE <sup>16</sup>

**Molecular structure:**



**Molecular formula:**  $C_{18}H_{21}NO_3 \cdot H_3PO_4 \cdot \frac{1}{2}H_2O$ .

**Chemical name:** 7, 8-Didehydro-4, 5 $\alpha$ -epoxy-3-methoxy-17-methylmorphinan-6 $\alpha$ -ol phosphate (1:1) (salt) hemihydrates.

**Molecular weight:** 406.4

**CAS No:** 76-57-3

**Physical appearance:** white crystalline powder, odorless and bitter in taste.

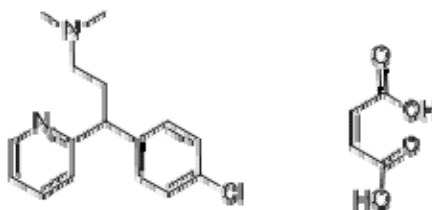
**Solubility:** soluble in water.

**pKa:** 8.21

**Category:** Antitussive & Analgesic.

### 2.1.2 CHLORPHENIRAMINE MALEATE <sup>17</sup>

**Molecular structure:**



**Chlorpheniramine Maleate**

**Molecular formula:**  $C_{20}H_{23}ClNO_4$

**Chemical name:** [3-(4-chlorophenyl)-3-(pyridin-2-yl)propyl]

dimethylamine.

**Molecular weight:** 390.86

**CAS No:** 132-22-9

**Physical appearance:** Odorless white crystalline solid or white powder with a bitter taste.

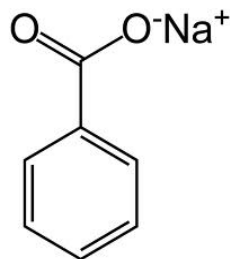
**Solubility:** soluble in water.

**pKa:** 4.21

**Category:** Antipruritic, Anti-Allergic Agent, Histamine (H<sub>1</sub>)Antagonist.

### 2.1.3 SODIUM BENZOATE <sup>18</sup>

**Molecular structure:**



#### **Sodium Benzoate**

**Molecular formula:** C<sub>6</sub>H<sub>5</sub>COONa (C<sub>7</sub>H<sub>5</sub>NaO<sub>2</sub>)

**Molecular weight:** 144.12

**CAS No:** 532-32-1

**Appearance:** White, odorless granules, pellets, or powder

**Solubility:** in water and in ethanol.

**pKa:** 8.0

**Category:** Preservative and pH stabilizer.

## 2.2 LITERATURE REVIEW:

1. Fuad Al-Rimawi *et al.*, (2010)<sup>19</sup> developed and validated Normal-phase LC method for simultaneous analysis of Pseudoephedrine hydrochloride, Dextromethorphan hydrobromide, Chlorpheniramine maleate and Paracetamol in tablet formulations and Separation was achieved on a silica column (5  $\mu$ m, 125  $\times$  4.6 mm inner diameter) using a mobile phase consisting of methanol/ammonium dihydrogen phosphate buffer (90:10, v/v) at a flow rate of 1.0 ml/min and UV detection at 220 nm. This new method is validated in accordance with USP requirements for new methods for assay determination, which include accuracy, precision, selectivity, linearity and range, robustness and ruggedness.
2. Mukesh Maithani *et al.*, (2010)<sup>20</sup> developed reverse phase high performance liquid chromatographic method for the simultaneous determination of chlorpheniramine maleate and phenylephrine in tablet dosage forms. A reversed-phase C-18 column (250 mm  $\times$  8 mm i.d., particle size 10  $\mu$ m) column with mobile phase consisting of acetonitrile and phosphate buffer 55:45 (v/v) (pH 5.6  $\pm$  0.02, adjusted with triethylamine) was used. The flow rate was 1.0 ml/min and effluents were monitored at 255 nm. The retention times of chlorpheniramine maleate and phenylephrine were found to be

3.13 min and 4.58 min, respectively. The method was validated in terms of linearity, range, specificity, accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ).

3. Adnan Manassra *et al.*, (2009)<sup>21</sup> proposed an HPLC method using UV detection for the simultaneous determination of pseudoephedrine hydrochloride, codeine phosphate and triprolidine hydrochloride in liquid formulation. C18 column (250 mm × 4.0 mm) is used as the stationary phase with a mixture of methanol: acetate buffer: acetonitrile (85:5:10, v/v) as the mobile phase. The factors affecting column separation of the analytes were studied. The calibration graphs exhibited a linear concentration range of 0.06–1.0 mg/ml for pseudoephedrine hydrochloride, 0.02–1.0 mg/ml for codeine phosphate, and 0.0025–1.0 mg/ml for triprolidine hydrochloride for a sample size of 5 µl with correlation coefficients of better than 0.999 for all active ingredients studied. The results demonstrate that this method is reliable, reproducible and suitable for routine use.
4. Ezzat M. Abdel-Moety *et al.*, (2009)<sup>22</sup> developed a selective and stability-indicating RP HPLC method for the simultaneous precise determination of promethazine and sodium benzoate even in the presence of main oxidative degradation products of promethazine. Good separation can be achieved on a C-18 column by using

mobile phase composed of 0.008M ammonium acetate and methanol (52:48v/v) by adopting isocratic elution programmed with UV detection at 253 nm. The method was linear over the concentration ranges of 4-60  $\mu\text{g ml}^{-1}$  & 0.2-3  $\text{mg ml}^{-1}$ , with accuracy of  $100.68 \pm 1.49\%$  and  $101.82 \pm 0.91\%$  and limits of quantitation (LOQ) of 0.8  $\mu\text{g ml}^{-1}$  & 20  $\mu\text{g/ml}$  for promethazine and sodium benzoate in orders.

5. Aravindaraj Joghee Rajua *et al.*, (2007) <sup>23</sup> developed and validated a convenient liquid chromatographic-single Quadrupole mass spectrometric (LC-MS) method for dexchlorpheniramine maleate (INN name: chlorpheniramine) determination in human plasma. The linearity was also excellent over the range of 1 to 150ng/ml of dexchlorpheniramine maleate concentration. The method was statistically validated for its selectivity, linearity, precision and robustness. This method was successfully applied to the analysis of chlorpheniramine maleate in clinical studies.
6. Maria-Elisa Capella-Peiro *et al.*, (2006) <sup>24</sup> optimized Capillary zone electrophoresis method to quantitatively determine codeine and paracetamol via central composite factorial design. Optimum separation conditions were achieved using phosphate buffer 20 mM (pH 6.8) and voltage (15 kV). The optimized procedure easily determined codeine and paracetamol with separation in less



than 3 min. Calibration curves ( $R > 0.999$ ) were prepared, with LODs of 13.5 and 340 ng mL<sup>-1</sup> for codeine and paracetamol, respectively, and a good R.S.D% (<3%). This method was applied to determine codeine and paracetamol in pharmaceutical formulations; recoveries coincided with stated contents.

7. María R. Gomez *et al.*, (2005)<sup>25</sup> described a simple, accurate and rapid method for the separation and simultaneous determination of codeine, diphenhydramine, ephedrine and noscapine present in cough–cold syrup formulations by capillary zone electrophoresis. Separations were carried out in less than 10 min with a 20 mM sodium tetraborate buffer, pH 8.50. The carrier electrolyte gave baseline separation with good resolution, great reproducibility and accuracy.
8. V. Galli *et al.*, (2004)<sup>26</sup> proposed a High-performance liquid chromatographic analysis of dextromethorphan, guaifenesin and benzoate in a cough syrup for stability testing. Forced degradation was also studied to demonstrate that the method could be employed during a stability study of the syrup. Final conditions were phosphate buffer (25 mM, pH 2.8) with triethylamine (TEA): acetonitrile (75:25, v/v). In such conditions, all the actives, excipients and degradation products were baseline resolved in less

than 14 min, and different wavelengths were used for the different analytes and related compounds.

9. Mohsen Kompany-Zareh *et al.*, (2004) <sup>27</sup> carried out spectrophotometric resolution of ternary mixtures of pseudoephedrine hydrochloride, dextromethorphan hydrobromide and sodium benzoate in syrups using wavelength selection by net analyte signals calculated with hybrid linear analysis (HLA). HLA was applied because it was simpler to adapt to the NAS regression plot methodology, and also used less factors than partial determinations. The method was successfully applied for the determination of pseudoephedrine HCl, dextromethorphan HBr, and sodium benzoate in cough suppressant syrup samples.
- 10.A Garcia *et al.*, (2003) <sup>28</sup> utilized Polyethylene glycol column for the determination of acetaminophen, phenylephrine and chlorpheniramine in pharmaceutical formulations with Supelco Discovery HS PEG column poly (ethylene glycol) 15×0.46 cm, 5 µm. The mobile phase was 20 mM phosphate buffer, pH 7.0–acetonitrile (90:10, v/v) at a flow-rate of 1 ml/min. UV detection was performed at 215 nm for all the compounds except acetaminophen, which was measured at 310 nm. Validation parameters permit us to consider this method suitable.

11.Darryl J. Hood *et al.*, (2002) <sup>29</sup> developed a simple, accurate and precise reversed phase HPLC method for rapid and simultaneous quantification of codeine phosphate, ephedrine HCl and Chlorpheniramine maleate in a cough–cold syrup formulation. Separations were carried out on a Zorbax® XDB C8 column (150×4.6 mm ID), 5 µm particle size. A gradient elution system was developed using varying percentages of two mobile phases: methanol: glacial acetic acid: triethylamine (980:15:6 v/v) and water–glacial acetic acid–triethylamine (980:15:6 v/v) with run time in less than 7 min with a flow rate of 1.5 ml/min and detected at a wavelength of 254 nm. The method was validated and met all analysis requirements of quality assurance and quality control recommended by FDA of the USA.

12.A.Marín *et al.*, (2002) <sup>30</sup> proposed the Validation of a HPLC quantification of acetaminophen, phenylephrine and chlorpheniramine in pharmaceutical formulations: capsules and sachets. Final chromatographic conditions were a gradient elution, being solvent A: phosphate buffer 40 mM at pH 6.0 and solvent B: acetonitrile. UV detection was performed at 215 nm for phenylephrine and chlorpheniramine, because at this wavelength sensitivity was higher than in other more characteristic wavelengths and it were necessary for the detection of minor

compounds. For acetaminophen 280 nm was employed. Validation parameters permit to consider the method adequate.

13. Murat Kartal *et al.*, (2001)<sup>31</sup> proposed LC method for the analysis of paracetamol, caffeine and codeine phosphate in pharmaceutical preparations. Paracetamol, caffeine and codeine phosphate were separated using a  $\mu$ Bondapak C<sub>8</sub> column by isocratic elution with flow rate 1.0 ml/min. The mobile phase composition was 420/20/30/30 (v/v/v/v) 0.01M KH<sub>2</sub>PO<sub>4</sub>, methanol, acetonitrile, isopropyl alcohol and spectrophotometric detection was carried out at 215 nm. The linear range of detection for paracetamol, caffeine and codeine phosphate was between 0.400 and 1500  $\mu$ g/ml; 0.075 and 90  $\mu$ g/ml; 0.300 and 30  $\mu$ g/ml respectively. The method has been shown to be linear, reproducible, specific, sensitive and rugged.

14. Ruby Ragonese *et al.*, (2000)<sup>32</sup> described the testing of a saturated factorial design using a full factorial design. Saturated factorial designs are often used to test the robustness of high-performance liquid chromatography (HPLC) methods. A full factorial design relies on fewer assumptions and hence could be used to evaluate the effectiveness of the saturated design. Both designs were used to test a gradient HPLC method for the assay of codeine phosphate, pseudoephedrine hydrochloride and Chlorpheniramine maleate. Six

HPLC conditions, including wavelength, mobile phase pH and ion pairing reagent concentration were tested using the saturated design. One interaction effect was indicated as a confounding effect by the saturated design and this was confirmed by the calculation of the same interaction effect using the full design. Overall the method was shown to be robust under the variety of HPLC conditions tested.

15. Io-Wah Lau *et al.*, (1995)<sup>33</sup> proposed an HPLC method using indirect conductometric detection for the simultaneous determination of eight active ingredients in cough-cold syrups. It involves the use of an Ultrasphere 5  $\mu\text{m}$  Spherical 80 Å Pore CN analytical column (250 mm  $\times$  4.6 mm) as the stationary phase with a mixture of water, acetonitrile and ethanol (38:60:2) containing 1 mM perchloric acid as the mobile phase. The active ingredients included bromhexine hydrochloride, chlorpheniramine maleate, codeine phosphate, dextromethorphan hydrobromide, diphenhydramine hydrochloride, ephedrine hydrochloride, papaverine hydrochloride and phenylephrine hydrochloride.

16. Brianne Weingarten *et al.*, (1995)<sup>34</sup> developed a rapid, reliable and rugged assay for determining codeine in human plasma using reversed-phase high-performance liquid chromatography with fluorescence detection. This analytical method utilized an ion-

exchange/mixed-mode solid-phase extraction procedure. The chromatographic separation was achieved using a  $150 \times 4.6$  mm I.D., 3- $\mu$ m reversed-phase C<sub>8</sub> (deactivated for basic analytes) column at ambient temperature. Fluorescence detection (excitation at 214 nm and emission above 345 nm) for codeine and nalorphine allowed for a detectable limit of 5 ng/ml. The results showed that the method was linear from 10 to 300 ng/ml. The method had good reproducibility, precision, accuracy and recoveries of 91 and 90% for codeine and nalorphine, respectively. This method has been applied to study the pharmacokinetics of codeine in normal human subjects.

17.G. Santoni *et al.*, (1992) <sup>35</sup> developed a reversed-phase high-performance liquid chromatographic method for the simultaneous determination of aspirin, propyphenazone and codeine phosphate in an analgesic tablet formulation. The proposed method is also suitable for the determination of small quantities of salicylic acid. The elution was isocratic using two C-8 columns and methanol-water (45:55) as mobile phase with 1.4% acetic acid and 5 mM tetramethylammonium bromide.

18.Zhao Rong Chen *et al.*, (1989) <sup>36</sup> developed a novel high-performance liquid chromatographic method for the determination of codeine, norcodeine and morphine in plasma and urine. The

compounds were separated on a cyano column (15 cm×4.6 mm, 5 µm particle size) using a mobile phase of acetonitrile-triethylamine-distilled water (4:0.1:95.9, v/v) pH 3.1 and then determined by fluorescence detection. Calibration curves in the range 5–200 ng/ml for plasma and 0.1–10 µg/ml for urine were linear and passed through the origin. The imprecision and inaccuracy of the assay were less than 10% and the limits of detection were 2 ng/ml for all three compounds in human plasma.

19.O.W.Lau *et al.*, (1989) <sup>37</sup> proposed a simple, rapid and accurate method for the simultaneous determination of active ingredients in cough—cold mixtures using isocratic ion-pair RP-HPLC method with an octadecylsilane column as the stationary phase and methanol, water, tetrahydrofuran, phosphoric acid mixtures as mobile phase including sodium dioctylsulphosuccinate as the ion-pair agent. The pH of the mobile phase was adjusted to 4.6 by means of phosphoric acid and ammonium hydroxide solutions. The proposed method involves the simple dilution of the samples with the mobile phase and the addition of metoclopramide hydrochloride as the internal standard.

20.Ron Ginman *et al.*, (1985) <sup>38</sup> described a procedure for the simultaneous determination of codeine and ibuprofen in human plasma following the administration of the two substances in a

proposed combination dosage form. The two substances were extracted separately from plasma and then determined together by high-performance liquid chromatography (HPLC) using a fluorescence detector. The codeine was first extracted from alkalinized plasma with hexane: dichloromethane (2:1, v/v) and then washed with sodium hydroxide solution. The ibuprofen was then extracted with hexane from the plasma acidified with sulphuric acid. The organic layers were collected, evaporated to dryness and the reconstituted residue was subjected to HPLC. The detection limit for codeine was  $8 \mu\text{g l}^{-1}$  and for ibuprofen  $1 \text{ mg l}^{-1}$ .

From the literature review, there is no proposed method for the quantification of these three compounds, namely codeine phosphate, chlorpheniramine maleate and sodium benzoate in combination in a syrup formulation.



## 2.3 AIM AND OBJECTIVE

The literature review reveals that numerous works have been carried out on RP-HPLC, capillary zone electrophoresis, spectrophotometric methods for the quantification of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate either individually or in combination with other drugs. The combination of Codeine phosphate, Chlorpheniramine maleate active drugs and Sodium benzoate as preservative is used in pharmaceutical preparations as cough syrup. This formulation is marketed by M/s Vivimed labs ltd, Hyderabad as a cough syrup. Codeine phosphate acts as antitussive, Chlorpheniramine maleate acts as antihistaminic and Sodium benzoate acts as preservative and pH stabilizer in the formulation. The need for determination of Sodium benzoate in the present study is that it is physically and chemically compatible for syrup formulation which improves shelf life of product. In this respect, a method for the analysis of this combination is needed for simultaneous quantification of all three combined components in one step.

The present study describes the development of a new rapid, efficient and reproducible RP-HPLC method using gradient mobile phase for the analysis of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate preservative in combination in cough syrup that offers certain advantages in its simplicity and time saving and applicable in routine analysis. It also describes the development of validation work as per ICH Q2B guidelines recommended by the Food and Drug Administration (FDA) of the United States.

## 2.4 PLAN OF WORK

Aimed to develop analytical method development and validation for Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate in combination in a cough syrup formulation.

The plan of the proposed work includes the following steps:

- The extensive survey of literature for Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate regarding their physico-chemical properties and analytical methods. This forms the basis for the development of methods.
- To undertake solubility studies for the analytes codeine phosphate, chlorpheniramine maleate and sodium benzoate.
- Selection of suitable solvent for quantitative extraction of analyte present in the formulations. The solvent should be readily available, economical and of analytical grade.
- To develop initial chromatographic conditions by selection of suitable column and appropriate wavelength in UV for detection and optimization of the method.
- To validate analytical method developed as per the ICH Q2B guidelines.

### 3. EXPERIMENTAL DETAILS

#### 3.1 MATERIALS AND METHODS:

##### List of chemicals used:

Table No 3: Table showing list of the chemicals used.

S.No	Chemical name	Supplier's Name	Grade
1.	Codeine phosphate	Vivimed labs ltd, Hyderabad, India.	In house Working standard
2.	Chlorpheniramine maleate	Vivimed labs ltd, Hyderabad, India.	In house Working standard
3.	Sodium benzoate	Vivimed labs ltd, Hyderabad, India.	In house Working standard
4.	Diammonium hydrogen orthophosphate	S.D.fine chemicals limited, Mumbai, India.	LR
5.	Ortho phosphoric acid	Merck, Mumbai, India.	AR
6.	Lichrosolv HPLC grade water	Merck, Mumbai, India.	AR
7.	Acetonitrile	Merck, Mumbai, India.	AR(HPLC grade)
8.	Methanol	Merck, Mumbai, India.	AR(HPLC grade)

**List of instruments used:****Table No 4: Table showing list of the instruments used.**

S.No	Instruments	Model	Manufacturer	Software
1.	HPLC	Model 2695	Waters	Empower2
2.	HPLC-U.V detector	Model 2489	Waters Dual $\lambda$ absorbance detector	Nil
3.	Analytical balance	-----	Mettler Toledo	Nil
4.	Ultrasonicate water bath	-----	Ultrasonicate SE- COUS	Nil
5.	pH meter	LI 127	Elico, Hydrogen Electrode	Nil

### **3.2 METHOD DEVELOPMENT-**

#### **SELECTION OF DETECTOR WAVE LENGTH:**

The wave length selection is made at 254 nm since all the three compounds maximum absorbance in UV spectrum as reported in the literature is in between 254-258 nm.

#### **TRIAL&ERROR METHODS:**

##### **TRIAL -1:**

#### **CHROMATOGRAPHIC CONDITIONS:**

Column : Spherisorb C18 250×4.6mm, 5µm.

Detector : 254nm

Flow rate : 1.2ml/min

Injection volume : 50µl

Run time : 20min

Mobile Phase : Buffer (pH: 3.5): Acetonitrile: Methanol (40:40:20)

Chromatogram of trial no: 1 is as shown in Fig No: 2.

##### **TRIAL- 2:**

#### **CHROMATOGRAPHIC CONDITIONS:**

Column : Spherisorb C18, 150×4.6mm, 5µm.

Detector : 254nm

Flow rate : 1.2ml/min

Injection volume : 50µl

Run time : 20min

Mobile phase : Buffer (pH-3.0): Acetonitrile (50:50)

Chromatogram of trial no: 2 is as shown in Fig. No: 3.

**Trail-3: Final method****CHROMATOGRAPHIC CONDITIONS:**

Column : ZODIAC C18 150×4.6mm, 3.5µm.

Detector : 254nm

Flow rate : 1.5ml/min

Injection volume : 50µl

Run time : 25min

Column temperature : 30°C

Program : gradient

**Gradient program**

<b>Time (Minutes)</b>	<b>Solvent-A (%)</b>	<b>Solvent-B (%)</b>
0.01	95	5
5	90	10
10	60	40
15	60	40
20	95	5
25	95	5

Chromatogram of trial no: 3 final methods are as shown in Fig. No: 4.

### ANALYTICAL METHOD-OPTIMISATION

**Aim:** The present study is to develop a new reverse phase liquid chromatographic method for simultaneous determination of codeine phosphate, chlorpheniramine maleate, sodium benzoate in a syrup formulation.

**Table No. 5: Table showing Optimized Chromatographic Parameters.**

OPTIMIZED CHROMATOGRAPHIC CONDITIONS	
Mode of separation	Gradient elution
Mobile phase	Solvent-A: Phosphate buffer pH-2.3 Solvent-B: Acetonitrile
Column	Zodiac C-18, 3.5 $\mu$ , 150 $\times$ 4.6mm
Flow rate	1.5 mL/ min
Detection Wavelength	254 nm
Injection volume	50 $\mu$ l
Column oven temperature	Ambient(30°C)
Run time	25 min

**Table No.6: Table showing Gradient program of elution.**

Time (Minutes)	Solvent-A (%)	Solvent-B (%)
0.01	95	5
5	90	10
10	60	40
15	60	40
20	95	5
25	95	5

**Procedure:****Preparation of Buffer solution:**

Weighed accurately 3.3gm of Diammonium hydrogen orthophosphate and dissolved in 1000 ml of water and adjusted the pH to 2.3 with Orthophosphoric acid.

**Diluent:** HPLC grade water.

**Preparation of Standard Solution:**

Accurately weigh and transfer 50mg of Codeine phosphate, 20mg Chlorpheniramine maleate & 50mg of Sodium benzoate working standards into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent .

Further pipette 5 ml of the above stock solution into a 25ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Inject 50 µl of the standard solution into the chromatographic system and measure the area for the Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate peaks and calculate the %Assay by using the assay formula.

Chromatogram of standard solution is as shown in Fig No: 4

**Assay procedure:****Preparation of Sample Solution:**

Accurately pipette out 5ml of the sample (equivalent to 1.264gm/ml) into a 100ml volumetric flask and 70ml of diluent was added and mixed well and made up to the mark with diluent. Mix well and filter through 0.45µm filter.

Inject 50 µl of the sample solution into the chromatographic system and measure the area for the Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate peaks and calculate the %Assay by using the assay formula.

Chromatogram of sample solution is as shown in Fig No: 5.



**Assay formula:**

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Label Claim} \times 100$$

Where,

AT = Peak Area of sample solution.

AS = Peak Area of standard solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard.

**Acceptance criteria:** The limit of assay is in between the 98% - 102%.

The chromatograms are as shown in Fig. No: 6-8 and assay results are tabulated and are as shown in Table No: 8.

### 3.3 METHOD VALIDATION

**Definition:** Validation is a process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce meeting, its predetermined specifications and quality attributes.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below:

- Accuracy
- Precision
- Specificity
- Detection Limit
- Quantitation Limit
- Linearity & Range
- Robustness
- Ruggedness
- System suitability testing

**Table No: 7 Table showing Validation parameters and Requirements.**

S.No	Parameter	Requirements
1.	Accuracy	%Recovery.
2.	Precision	SD, %RSD.
3.	Linearity	Correlation coefficient, y-intercept, Slope of regression line.
4.	Range	80-120% of test solution.
5.	Specificity	No interference to be observed, Assay results unaffected.
6.	Robustness	%RSD
7.	Ruggedness	%RSD

**3.3.1 SYSTEM SUITABILITY TESTING:**

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated and the parameters like tailing factor, retention time, theoretical plates per unit, resolution factor are determined and the results are tabulated and are as shown in Table No: 9.

**3.3.2 ACCURACY:**

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. Accuracy may be inferred once precision, linearity, and specificity have been established.

**Procedure:****Preparation of Standard solution (100µg/ml):**

Accurately weigh and transfer 50mg of Codeine phosphate and 20mg of Chlorpheniramine maleate and 50mg of Sodium benzoate working standards into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 5 ml of the above stock solution into a 25ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

**Preparation of 50% sample solution:**

Accurately weigh and transfer 25mg of Codeine phosphate and 10 mg of Chlorpheniramine maleate and 25mg of Sodium benzoate into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 5ml of the above stock solution into a 25ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45 $\mu$ m filter.

**Preparation of 100% solution:**

Accurately weigh and transfer 50mg of Codeine phosphate and 20mg of Chlorpheniramine maleate and 50mg of Sodium benzoate working standards into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 5 ml of the above stock solution into a 25ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45 $\mu$ m filter.

**Preparation of 150% solution:**

Accurately weigh and transfer 75 mg of Codeine phosphate and 30 mg of Chlorpheniramine maleate and 75mg of Sodium benzoate API sample into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 5ml of the above stock solution into a 25ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45 $\mu$ m filter.

Inject 50 $\mu$ l of placebo and standard solutions of Accuracy -50%, Accuracy -100% and Accuracy -150% solutions into HPLC. Now calculate the amount obtained and amount added (API) for Codeine phosphate and Chlorpheniramine maleate and Sodium benzoate samples. Calculate the concentration in  $\mu$ g/ml in the spiked placebo in all the

above cases by comparing with the standard solution. Calculate the individual recovery and mean recovery values. The chromatograms are as shown in Fig. No: 9-12 and the results are tabulated and shown in Table No: 10.

**Formula:**

$$\% \text{recovery} = \frac{(\text{Amount recovered})}{(\text{Actual amount added})} \times 100$$

**Acceptance criteria:**

Percentage recovery in all the cases should be between 98.0 and 102.0 %.

**3.3.3 PRECISION:**

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples using a minimum of 6 determinations at 100 percent of the test concentration. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

**Repeatability**

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

**Intermediate precision**

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment etc.

**Reproducibility**

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

**Procedure:****A) System Precision:****Preparation of Standard Solution (100 µg/ml):**

Accurately weigh and transfer 50mg of Codeine phosphate and 20mg of Chlorpheniramine maleate and 50mg of Sodium benzoate working standards into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 5 ml of the above stock solution into a 25ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Inject 50µl of the blank solution and the standard solution of 100 µg/ml for six times and calculate the %RSD for the area of six replicate injections. The chromatograms are as shown in Fig No: 13-19 and the results are tabulated shown in Table No: 11.

**B) Method Precision:****Preparation of Sample Solution:**

Accurately pipette out 5ml of the sample (equivalent to 1.264gm/ml) into a 100ml volumetric flask and 70ml of diluent was added and mixed well and made up to the mark with diluent. Mix well and filter through 0.45µm filter.

Inject 50µl of the blank solution and six replicate injections of sample solution of 100 µg/ml for six times and calculate the %RSD for the area of six replicate injections. The chromatograms are as shown in Fig No: 20 and the results are tabulated and are as shown in Table No: 11.

**%RSD Formula:  $(\sigma/\mu)*100$** 

**Acceptance criteria:** % Relative standard deviation (%RSD) for the areas of codeine phosphate, chlorpheniramine maleate and sodium benzoate from the standard chromatograms should not be more than 1.0 %.

**3.3.4 SPECIFICITY:**

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

**Procedure:****Preparation of Standard solution (100µg/ml):**

Accurately weigh and transfer 50mg of Codeine phosphate and 20mg of Chlorpheniramine maleate and 50mg of Sodium benzoate working standards into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 5 ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Inject 50 µl of blank solution into the HPLC system and chromatograph.

Inject 50 µl of Standard solution for six times into the HPLC system and chromatograph.

Compare the chromatograms visually and check for any interference. Calculate the average and relative standard deviation of area of codeine phosphate, chlorpheniramine maleate and sodium benzoate from standard chromatograms shown in Fig No: 21-27 and the results are tabulated and are as shown in Table No: 12.

%RSD for the area of six replicate injections is to be calculated using formula.

**%RSD Formula:  $(\sigma/\mu)*100$**

**Acceptance criteria:**

There should not be any peak in the blank and Placebo solution run at the retention time corresponding to codeine phosphate, chlorpheniramine maleate and sodium benzoate as in standard run.



**LINEARITY&RANGE:****3.3.5 LINEARITY:**

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of correlation coefficient, y-intercept, slope of the regression line and residual sum of squares. A plot of the data should be included. For the establishment of linearity, a minimum of five concentrations is recommended.

**3.3.6 RANGE:**

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

**Procedure:****Preparation of Standard Stock Solution:**

Accurately weigh and transfer 50 mg of Codeine phosphate, 20 mg of Chlorpheniramine maleate and 50 mg of Sodium benzoate working standards into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it

completely and make volume up to the mark with the same solvent. (Stock solution)

**Preparation of (25, 50, 75, 100,125 and 150 µg/ml) sample solutions:**

From the above stock solution pipette out 2.5,5.0,7.5,10.0,12.5,15.0 ml respectively into individual 50ml of volumetric flasks and dilute up to the mark with diluent to prepare 25,50,75,100,125,150 µg/ml of sample solutions respectively. Mix well and filter through 0.45µm filter.

Inject 50µl of blank solution and each linearity level standard solutions into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The chromatograms corresponding to linearity are as shown in Fig No: 28-38 and the results are tabulated shown in Table No: 13-15.

**Formula:** The equation for the correlation coefficient is:

$$Correl(X,Y) = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

**Regression line equation:  $y=mx+c$**

Where, m = slope, c = y-intercept.

**Acceptance criteria for Linearity:**

Correlation coefficient should not be less than 0.995.

**Acceptance criteria for Range:**

RSD for the areas of Linearity level-1 solution should not be more than 1.0%.

RSD for the areas of Linearity level-6 solution should not be more than 1.0%.

**3.3.7 ROBUSTNESS:**

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate

variations in method parameters and provides an indication of its reliability during normal usage.

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition and pH were made to evaluate the impact on the method.

**A)** The flow rate was varied at 0.1 ml variation to normal flow rate of 1.5 ml.

**Preparation of Standard Solution (100µg/ml):**

Accurately weigh and transfer 50mg of Codeine phosphate and 20mg of Chlorpheniramine maleate and 50mg of Sodium benzoate working standards into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 5 ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Inject 50µl of the blank solution and the standard solution of 100 µg/ml for five times and analysed using varied flow rates (1.4 ml, 1.6 ml) along with method flow rate and calculate the %RSD for the area of five replicate injections. The chromatograms are as shown in Fig No: 39 and the results are tabulated shown in Table No: 16.

**B)** The pH of buffer solution in Mobile phase was varied at +/- 0.1 from normal pH of 2.3.

**Preparation of Standard Solution (100µg/ml):**

Accurately weigh and transfer 50mg of Codeine phosphate and 20mg of Chlorpheniramine maleate and 50mg of Sodium benzoate working standards into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 5 ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Inject 50 $\mu$ l of the blank solution and the standard solution of 100  $\mu$ g/ml for five times and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method and calculate the %RSD for the area of five replicate injections. The chromatograms are as shown in Fig No: 40 and the results are tabulated shown in Table No: 16.

**%RSD Formula:  $(\sigma/\mu)*100$**

**Acceptance criteria:**

Relative standard deviation (RSD) of areas of codeine phosphate, chlorpheniramine maleate and sodium benzoate from five standard chromatograms in all the flow rate variation and mobile phase composition should not be more than 1.0 %.

**3.3.8 RUGGEDNESS:**

Ruggedness of the current method was demonstrated by analyzing six samples (assay) of syrup formulation by two analysts in the same laboratory. The RSD for the 12 samples is calculated.

**Procedure:**

**Preparation of Standard Solution (100 $\mu$ g/ml):**

Accurately weigh and transfer 50mg of Codeine phosphate and 20mg of Chlorpheniramine maleate and 50mg of Sodium benzoate working standards into a 100 ml volumetric flask, add about 70 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 5 ml of the above stock solution into a 25 ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45 $\mu$ m filter.

Inject the blank solution and standard solution of 100  $\mu$ g/ml for six times and analysed by two analysts and calculate the %RSD for the area of twelve replicate

injections. The chromatograms are as shown in Fig No: 43, 44 and the results are tabulated shown in Table No: 17.

**%RSD Formula:  $(\sigma / \mu) * 100$**

**Acceptance criteria:**

Relative standard deviation (RSD) of areas of codeine phosphate, chlorpheniramine maleate and sodium benzoate from twelve standard chromatograms should not be more than 1.0 %.

## 4.1 RESULTS AND DISCUSSION

In the present work a new method development and validation was carried out for the estimation of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate by RP-HPLC technique. The wavelength selection was made at 254 nm since all the selected drugs reported in various literatures were having a maximum absorbance at around 254 to 258 nm. Hence the wavelength was selected at 254 nm for the detection of the three compounds.

**4.1.1 METHOD DEVELOPMENT:** For the method development several trials were carried out and reported. These lead to the optimized chromatographic conditions for the separation and estimation of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate in the bulk and marketed formulation. Preliminary studies involved trying ODS-silica columns and several mobile phase compositions for the effective separation of these three drugs. By Zodiac C-18, 3.5 $\mu$ ,150 $\times$ 4.6mm column eluted with Solvent-A: Phosphate buffer pH-2.3, Solvent-B: Acetonitrile by gradient elution pattern at a flow rate of 1.5 ml/ min and a detection wavelength of 254 nm with injection volume of 50  $\mu$ l at Ambient(30°C) temperature afforded the best separation of these analytes. The chromatograms of trial methods were as shown in Fig.No:3-5 and optimized method were as shown in Fig. No: 6-8. The assay results were as shown in Table No: 8.

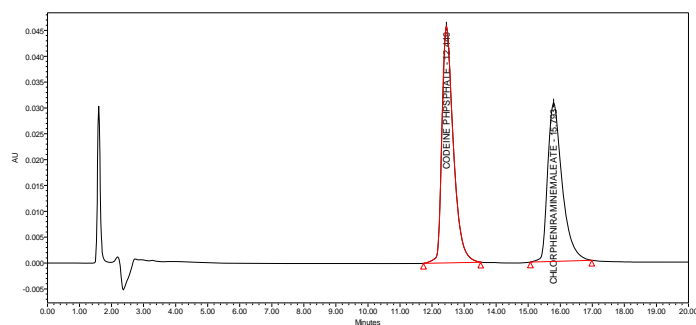
**Results of Trial-1:** RT'S were observed at 11.236(CP), 15.568(CPM) and the third peak is not eluted in 20 min runtime. Due to asymmetry in peaks and longer RT's another trial is made with change in mobile phase-buffer pH.

**Results of Trial-2:** RT's were observed at 10.236(CP), 13.568(CPM) and the third peak is not eluted in 20 min runtime. Due to tailing in peaks and to further reduce

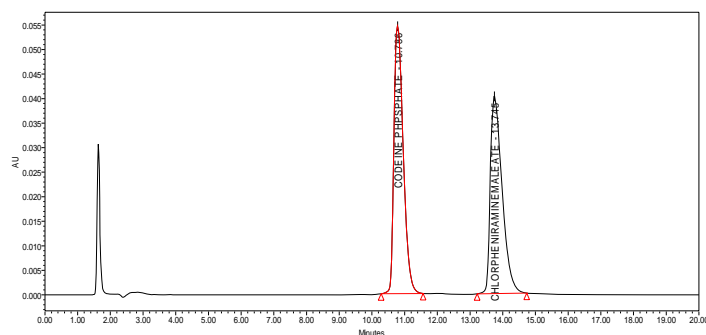
RT's another trial is made with change in mobile phase-buffer pH, flow rate and elution pattern.

**Results of Trial-3(Final Optimized Method):** RT's were observed at 7.169(CP), 9.480(CPM) and 10.860(SB). The peaks are sharply resolved with less tailing and hence the trial-3 method is optimized for analysis.

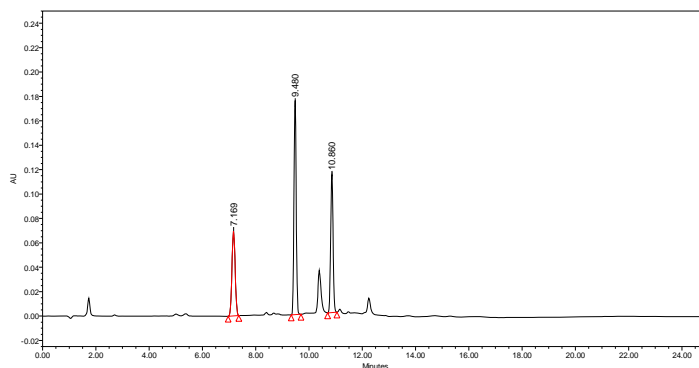
**Fig. No. 3: Chromatogram showing Trial-1.**



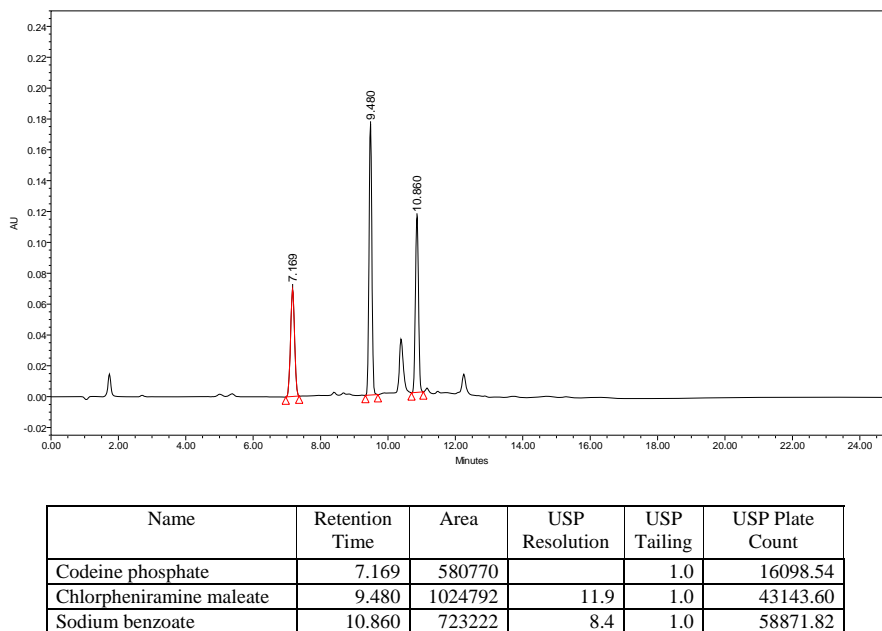
**Fig. No. 4: Chromatogram showing Trial-2.**



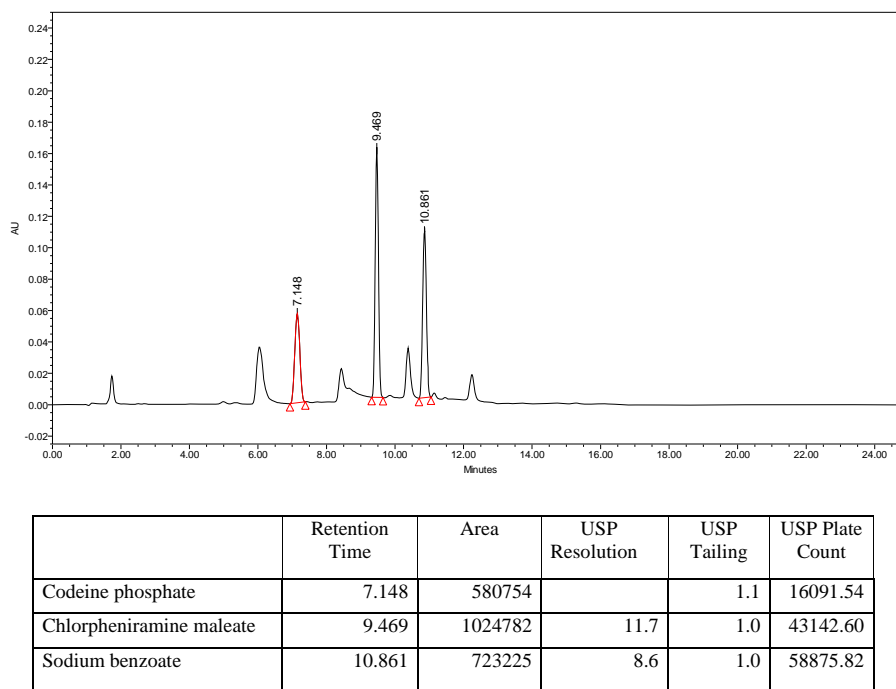
**Fig. No. 5: Chromatogram showing Trial-3.**



**Fig. No: 6 Chromatogram showing peaks of standard solution of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate.**

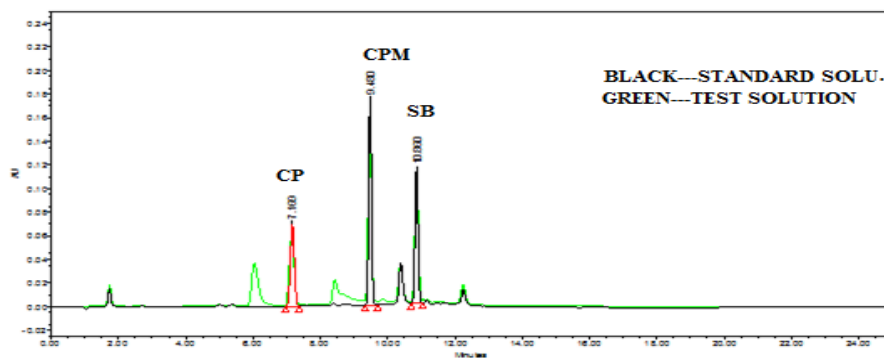


**Fig. No.7:Chromatogram showing peaks of test solution of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate.**





**Fig. No. 8:Chromatogram showing overlay of Standard and Test samples of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate.**



**Table No: 8 Table showing Assay Results of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate.**

S.No	Compound name	Assay value
1.	Codeine phosphate	99.2%
2.	Chlorpheniramine maleate	101.2%
3.	Sodium benzoate	99.8%

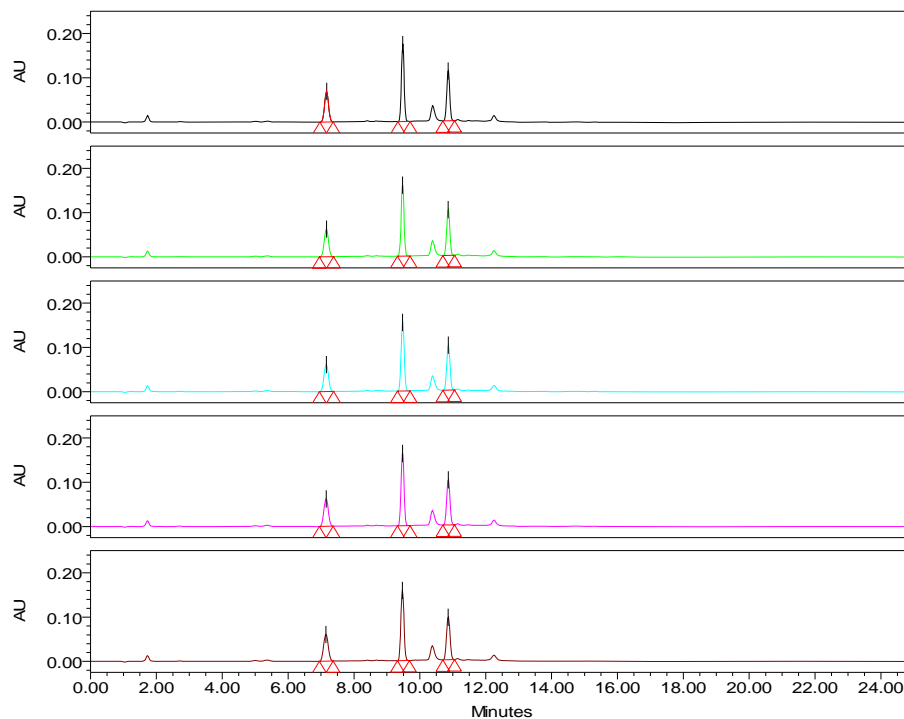
**4.1.2 METHOD VALIDATION:**

After method development, the validation of the current method has been performed in accordance with USP requirements for assay determination (Category-I: analytical methods for quantitation of active ingredients in finished pharmaceutical products) which include accuracy, precision, selectivity, linearity and range, robustness and ruggedness.

**4.1.2.1 System Suitability Testing:**

System suitability testing is an integral part of many analytical procedures. System suitability test parameters like tailing factor, retention time, theoretical plates per unit, resolution factor are determined and the results are tabulated and are as shown in Table No: 9.

**Fig. No. 9: Chromatograms showing system suitability testing (INTRA DAY STUDIES) of Standard Solutions of codeine phosphate, chlorpheniramine maleate and sodium benzoate.**



**Table No: 9 Table showing list of system suitability parameters of codeine phosphate, chlorpheniramine maleate and sodium benzoate.**

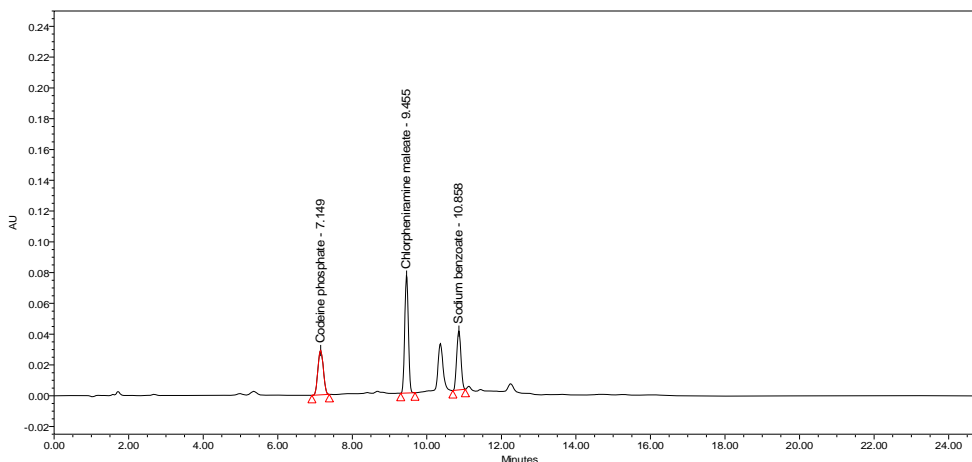
Parameters	Codeine phosphate	Chlorpheniramine maleate	Sodium benzoate
Tailing factor	1.2	1.2	1.1
Retention time	7.18	9.39	10.92
Theoretical plates per unit	16779.2	43624.9	47472.4
Resolution		2.9	2.02

#### 4.1.2.2 Accuracy (Recovery):

Accuracy of the method was studied by preparing the placebo of the syrup formulation according to the formulation procedure. To the required quantity of placebo, a known quantity of the three active ingredients (codeine phosphate, chlorpheniramine maleate and sodium benzoate) with the same proportion as in the

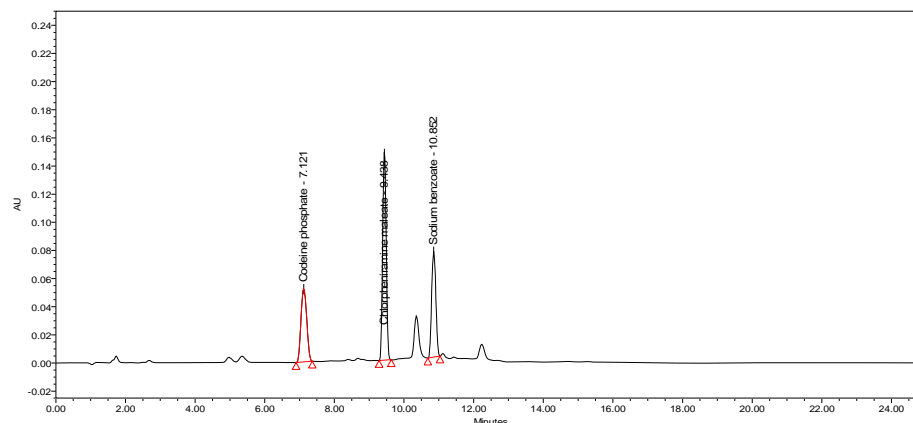
drug formulation was added to get simulated drug formulation. The chromatograms are as shown in Fig. No: 10-12. The results are as shown in the Table No: 10 and the mean % recovery of the assay is within  $100 \pm 2.0\%$  for each ingredient. These results show that the method was accurate.

**Fig. No. 10: Chromatogram showing Accuracy 50% of codeine phosphate, chlorpheniramine maleate and sodium benzoate.**



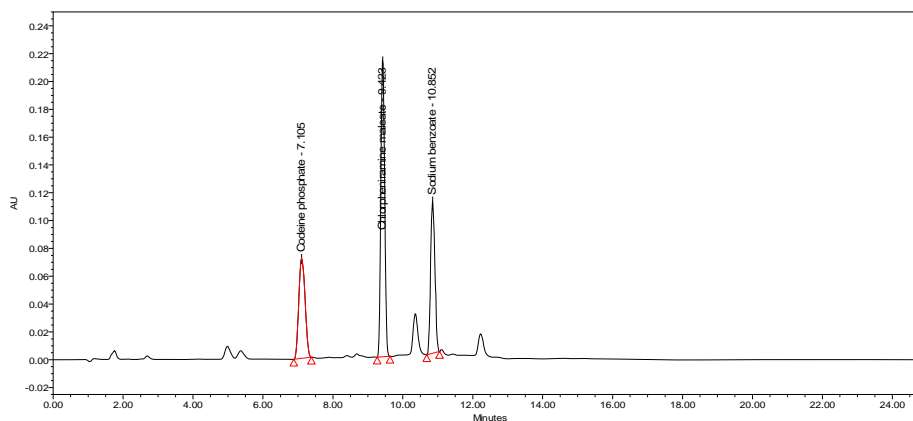
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.149	593890		1.1	11267.64
Chlorpheniramine maleate	9.455	1053085	10.1	1.0	43121.69
Sodium benzoate	10.858	727773	7.3	1.0	52097.67

**Fig.No. 11: Chromatogram showing Accuracy -100% of codeine phosphate, chlorpheniramine maleate and sodium benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.121	593882		1.1	11265.58
Chlorpheniramine maleate	9.438	1053076	10.3	1.0	45121.72
Sodium benzoate	10.852	727779	7.4	0.9	56097.54

**Fig. No. 12: Chromatogram showing Accuracy – 150% of codeine phosphate, chlorpheniramine maleate and sodium benzoate.**



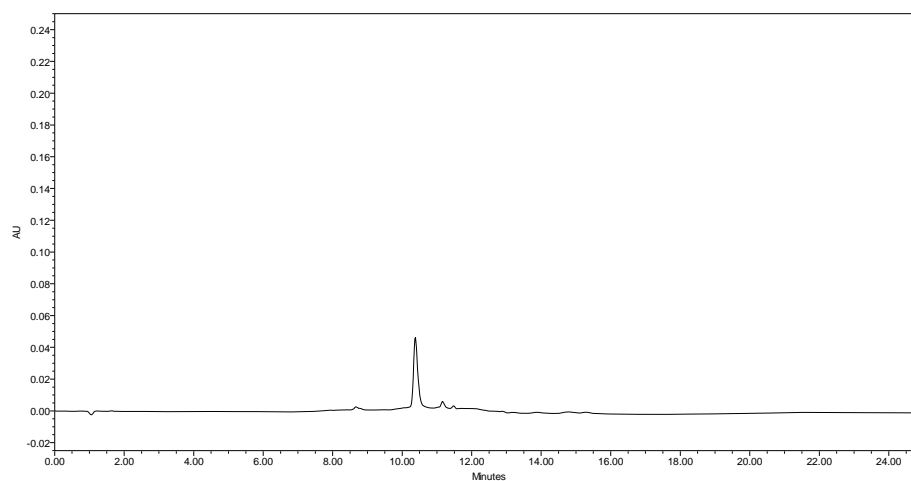
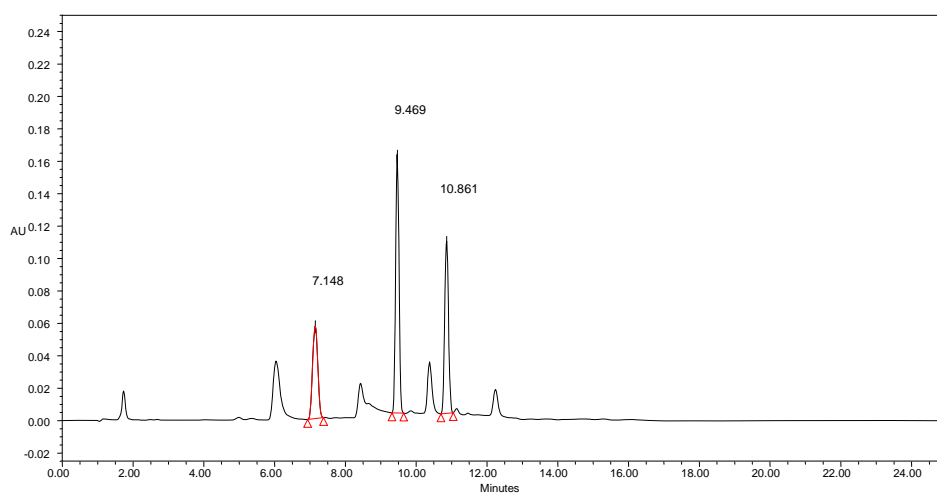
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.105	593886		1.1	11264.52
Chlorpheniramine maleate	9.430	1053082	10.5	1.0	45123.76
Sodium benzoate	10.852	727765	7.1	1.0	46095.58

**Table no: 10: Table showing results of % Recovery studies for Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**

Inj.sample	Spike level	Amount present	Amount recovered	% recovered	Mean recovery	Acceptance Criteria
<b>Codeine Phosphate</b>	50 %	25mg	24.8mg	99.2%	98.7%	100 ± 2.0%
	100 %	50mg	49.8mg	99.6%		
	150 %	75mg	75.07mg	100.1%		
<b>Chlorpheniramine Maleate</b>	50 %	10mg	9.85mg	98.5%	98.5%	100 ± 2.0%
	100 %	20mg	19.7mg	98.5%		
	150 %	30mg	29.16mg	98.6%		
<b>Sodium Benzoate</b>	50 %	25mg	24.72mg	98.9%	99.1%	100 ± 2.0%
	100 %	50mg	49.35mg	98.7%		
	150%	75mg	74.4mg	99.2%		

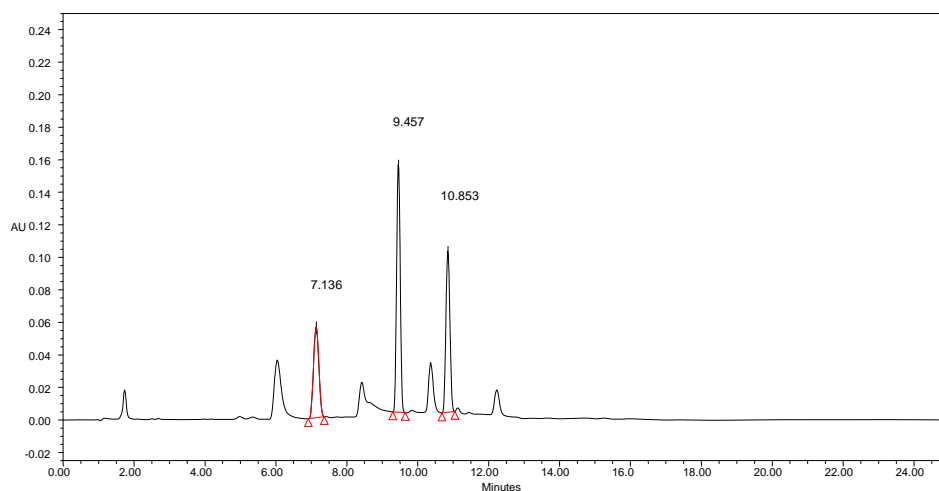
#### 4.1.2.3 Precision:

The system precision of this method was evaluated by calculating the %RSD of the peak areas of six replicate injections of the standard solution, which were found to be 0.41%, 0.35% and 0.37% and for method precision evaluated with six sample replicate injections were found to be 0.43%, 0.36% and 0.39% for codeine phosphate, chlorpheniramine maleate and sodium benzoate respectively and it was found to be less than 1.0% shown in the Table No: 11. The chromatograms were as shown in Fig. No: 13-20. These results show that the current method is repeatable and reproducible.

**Fig.No.13: Chromatogram showing Precision-blank solution.****Fig.No.14: Chromatogram showing Precision of Test sample Preparation-1 for codeine phosphate, chlorpheniramine maleate and sodium benzoate.**

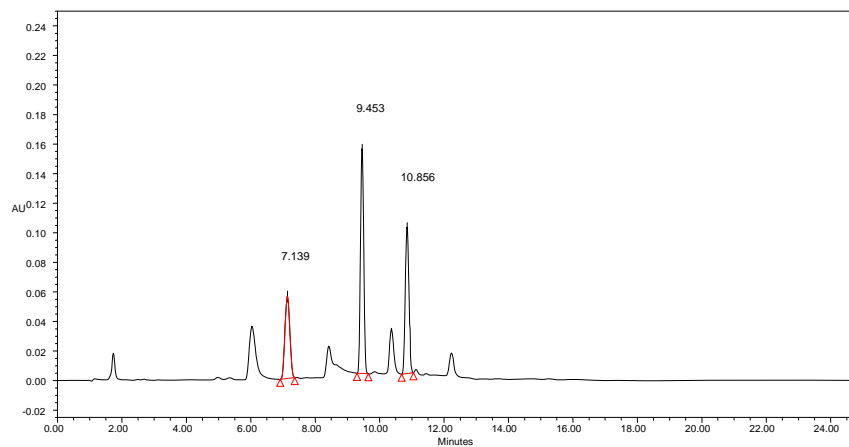
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.148	593894		1.1	11261.55
Chlorpheniramine maleate	9.469	1053092	10.3	1.0	43123.64
Sodium benzoate	10.861	727768	7.5	1.0	46095.47

**Fig. No15: Chromatogram showing Precision of Test sample Preparation-2 for codeine phosphate, chlorpheniramine maleate and sodium benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.136	593890		1.1	11263.95
Chlorpheniramine maleate	9.457	1053086	10.2	1.0	45125.84
Sodium benzoate	10.853	727754	7.3	1.0	46092.54

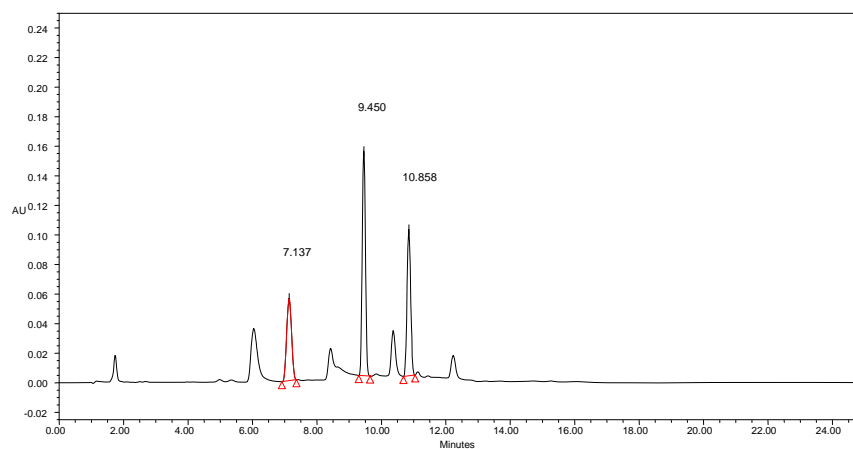
**Fig. No.16: Chromatogram showing Precision of Test sample Preparation-3 for codeine phosphate, chlorpheniramine maleate and sodium benzoate.**





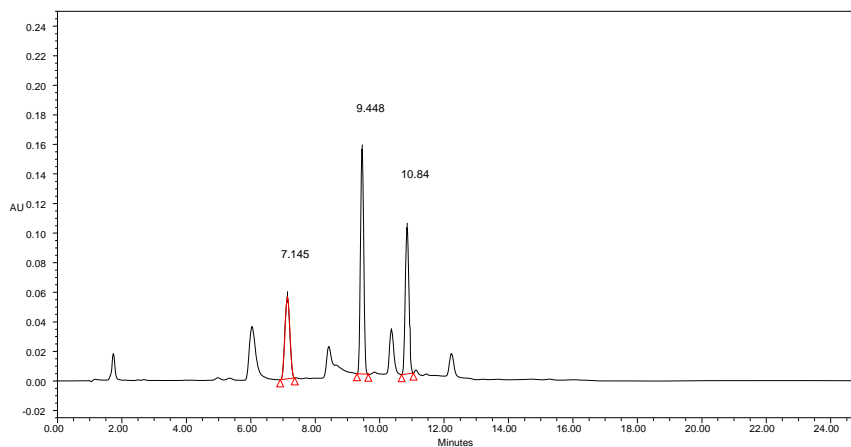
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.139	593887		1.1	11266.85
Chlorpheniramine maleate	9.453	1053095	10.3	1.0	43127.64
Sodium benzoate	10.856	727751	7.4	1.0	45095.52

**Fig. No. 17: Chromatogram showing Precision of Test sample Preparation-4 for codeine phosphate, chlorpheniramine maleate and sodium benzoate.**



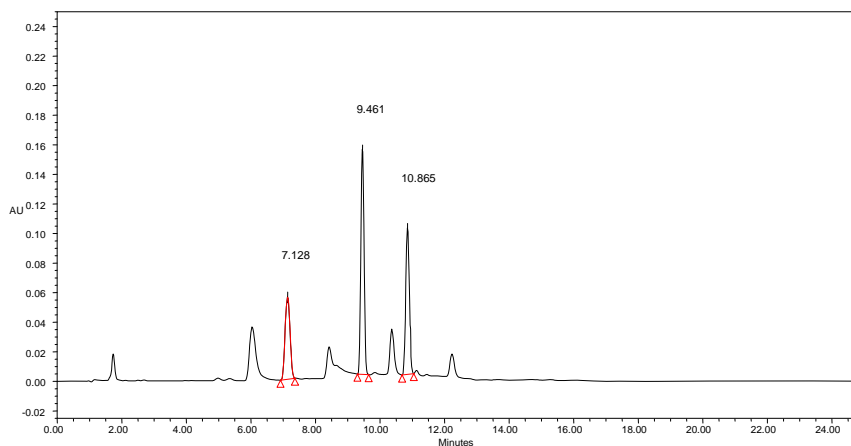
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.137	593887		1.1	11266.75
Chlorpheniramine maleate	9.450	1053092	10.4	1.0	43129.94
Sodium benzoate	10.858	727762	7.2	1.0	45087.64

**Fig. No.18: Chromatogram showing Precision of Test sample Preparation-5 for codeine phosphate, chlorpheniramine maleate and sodium benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.145	593896		1.1	11273.65
Chlorpheniramine maleate	9.448	1053072	10.3	1.0	43135.84
Sodium benzoate	10.848	727748	7.1	1.0	44082.74

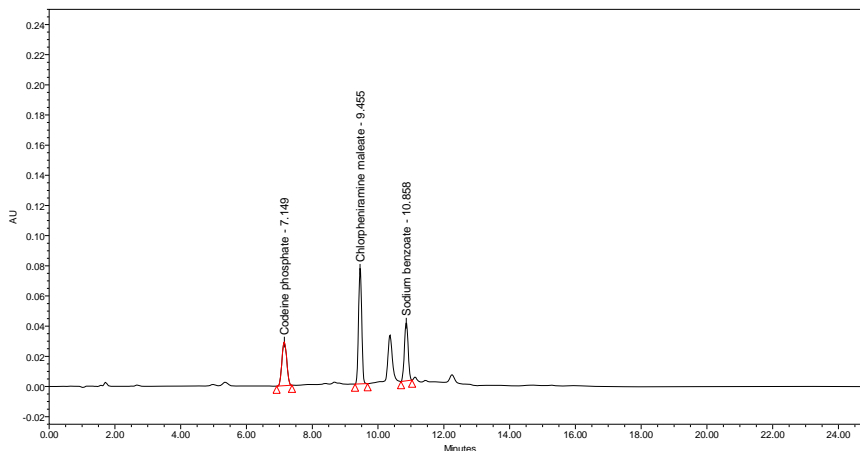
**Fig. No.19: Chromatogram showing Precision of Test sample Preparation-6 for codeine phosphate, chlorpheniramine maleate and sodium benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.128	593898		1.1	11283.85
Chlorpheniramine maleate	9.461	1053092	10.1	1.0	45135.54
Sodium benzoate	10.865	727765	7.2	1.0	46082.94

### Method Precision

**Fig.No.20: Chromatogram showing Method Precision of Codeine phosphate, Chlorpheniramine maleate, and Sodium Benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.149	593880		1.1	11273.89
Chlorpheniramine maleate	9.455	1053076	10.2	1.0	45095.84
Sodium benzoate	10.858	727744	7.2	1.0	46097.56

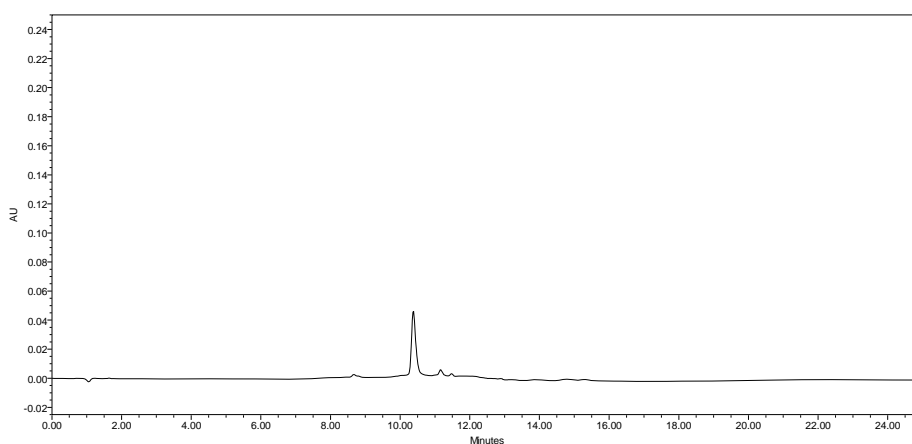
**Table No: 11 Table showing Results of Precision for Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**

Parameter	System Precision			Method Precision		
Average Area	Codeine phosphate	Chlorpheniramine maleate	Sodium benzoate	Codeine phosphate	Chlorpheniramine maleate	Sodium benzoate
	582985	1036039	719061	580118	1036157	713987
SD	2390.2	3724.3	2719.3	2494.5	3812.9	2834.8
%RSD	0.41	0.35	0.37	0.43	0.36	0.39

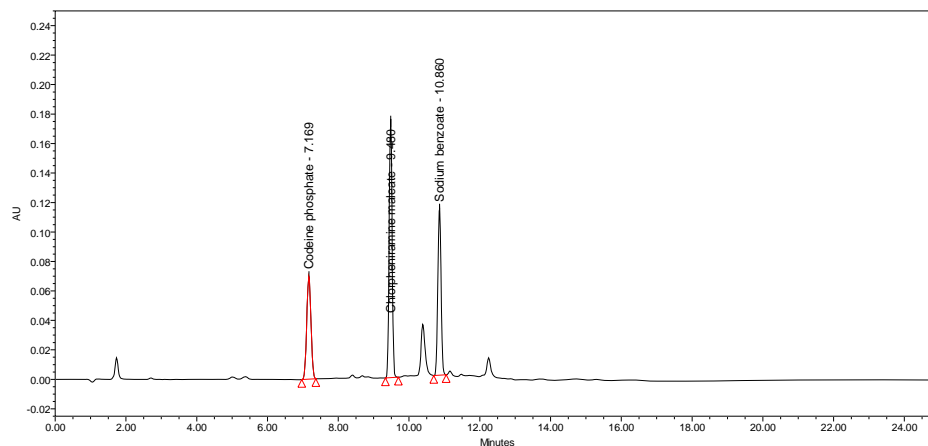
#### 4.1.2.4 Specificity/Selectivity:

Selectivity of the current method was demonstrated by good separation of the three analytes from each other, see Fig No: Furthermore, excipients of the formulation did not interfere with the active ingredients of the drug product, as shown in the chromatograms. The average and relative standard deviation of area from standard chromatograms was calculated. The chromatograms are as shown in Fig. No:21-27 and the results are as shown in Table No: 12

**Fig. No. 21: Chromatogram showing Specificity- Blank solution**

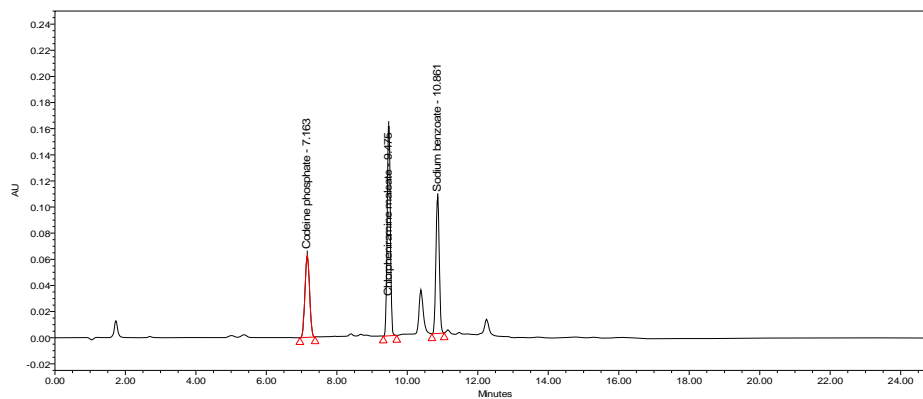


**Fig. No. 22: Chromatogram showing Specificity-Standard-1 solution of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



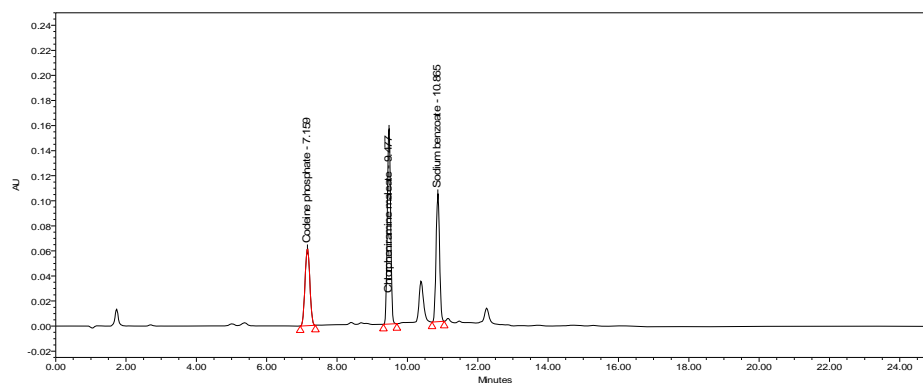
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.169	580770		1.0	16098.54
Chlorpheniramine maleate	9.480	1024792	11.9	1.0	43143.60
Sodium benzoate	10.860	723222	8.4	1.0	53871.82

**Fig. No. 23: Chromatogram showing Specificity- Standard-2 solution of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



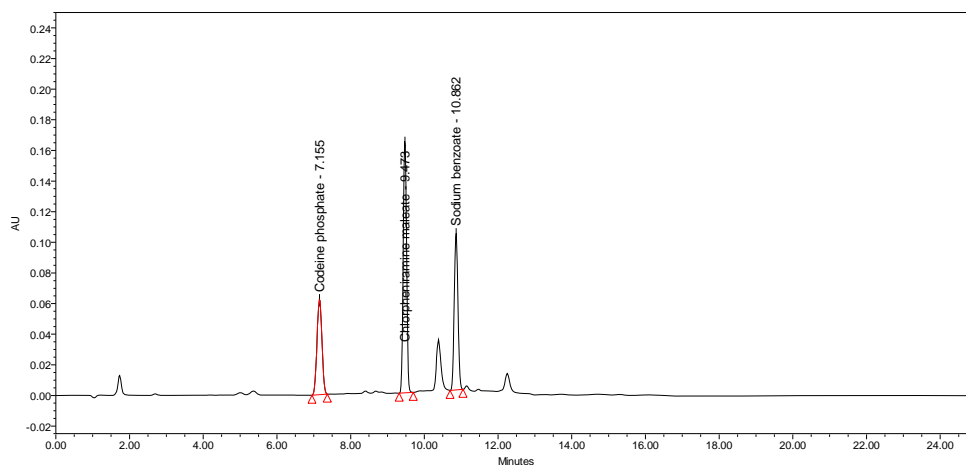
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.163	566151		1.0	13652.01
Chlorpheniramine maleate	9.475	1023254	11.2	1.0	51711.86
Sodium benzoate	10.861	699694	8.0	1.0	57803.38

**Fig.No.24: Chromatogram showing Specificity- Standard-3 solution of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



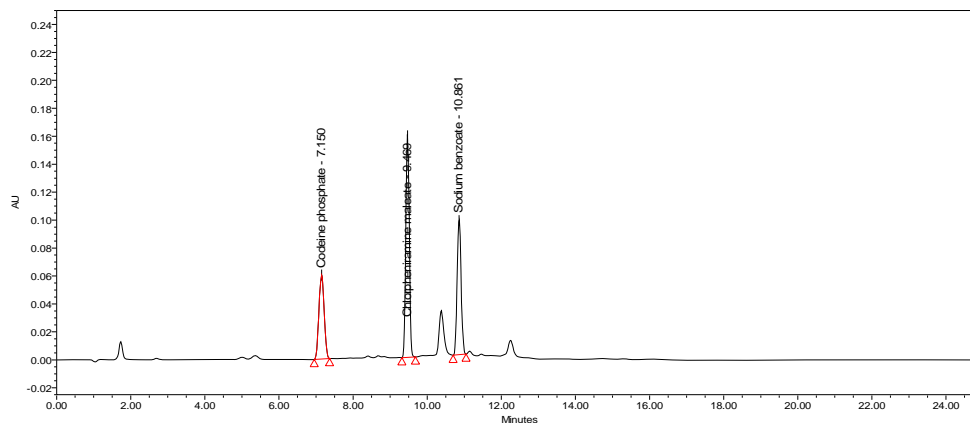
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.159	584035		1.0	12409.89
Chlorpheniramine maleate	9.477	1031721	10.6	1.0	45450.64
Sodium benzoate	10.865	719025	7.5	1.0	51921.63

**Fig. No. 25: Chromatogram showing Specificity- Standard-4 solution of Codeine phosphate, Chlorpheniramine maleate, and Sodium Benzoate.**



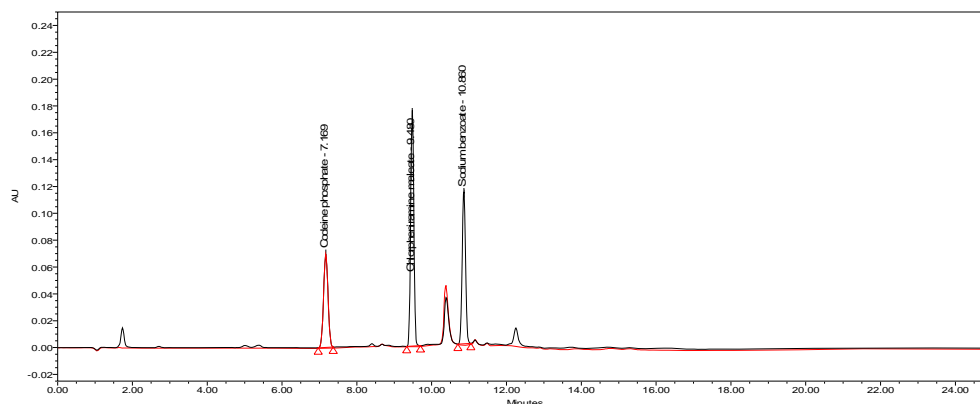
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.155	590077		1.0	12264.39
Chlorpheniramine maleate	9.473	1047339	10.8	1.0	48743.16
Sodium benzoate	10.862	725587	7.6	1.0	51955.02

**Fig. No. 26: Chromatogram showing Specificity- Standard-5 solution of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.150	593892		1.0	11267.77
Chlorpheniramine maleate	9.469	1053093	10.3	1.0	45121.66
Sodium benzoate	10.861	727778	7.2	1.0	46097.62

**Fig. No. 27: Chromatogram showing Blank & Standard solution-1 overlay report of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



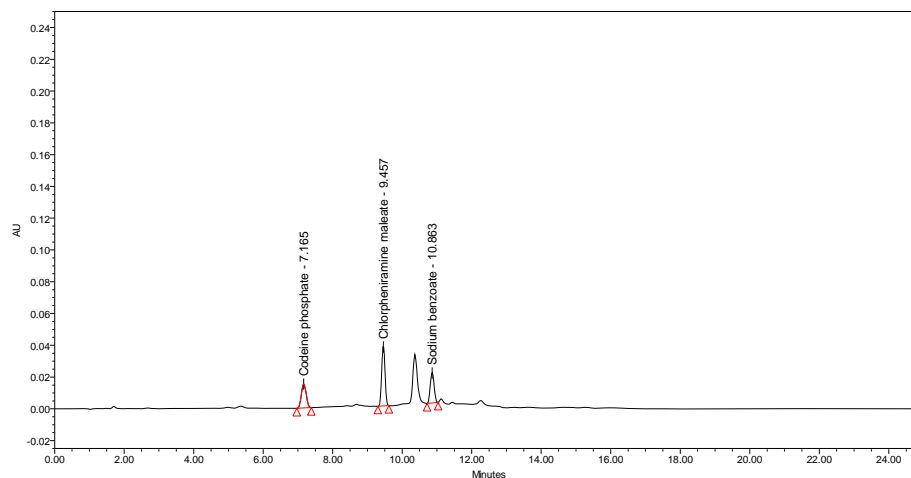
**Table No: 12 Table showing results of Specificity of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**

Parameter	Codeine phosphate	Chlorpheniramine maleate	Sodium benzoate
Average area	582964	1036045	719072
SD	2389.5	3724.7	2719.5
%RSD	0.40	0.38	0.39

#### 4.1.2.5 & 4.1.2.6 Linearity and range:

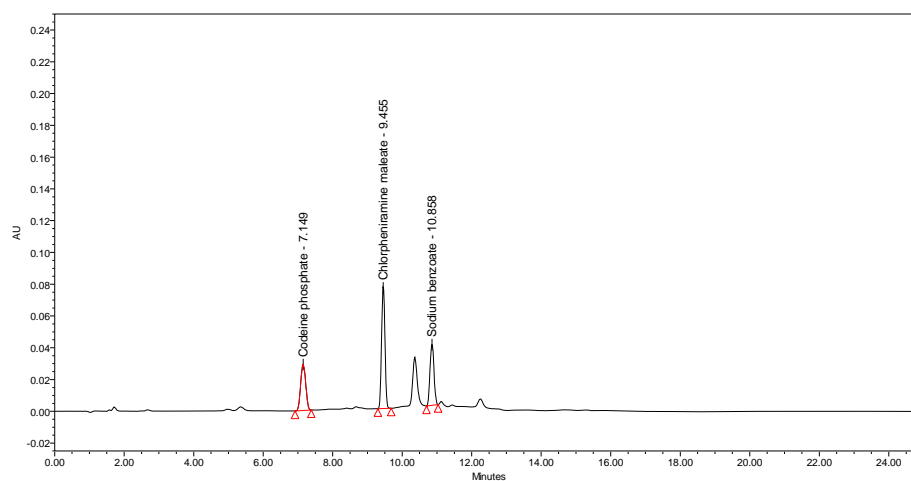
Linearity was studied from different concentrations of the three analytes in the range of 12.5-75 µg/ml for codeine phosphate, 5-30 µg/ml for chlorpheniramine maleate and 12.5-75 µg/ml for sodium benzoate and the linearity between the peak-area and the concentration was examined for each analyte. The chromatograms are as shown in Fig.No:28-38. The results obtained are shown in the Table No: 13-15 and show that the current method was linear for the three analytes in the range specified above with a correlation coefficient of better than 0.999.

**Fig. No.28: Chromatogram showing Linearity level-1 (25%) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.165	146517		1.1	2666.65
Chlorpheniramine maleate	9.457	252347	10.2	1.0	10129.86
Sodium benzoate	10.863	147150	7.1	1.1	11087.68

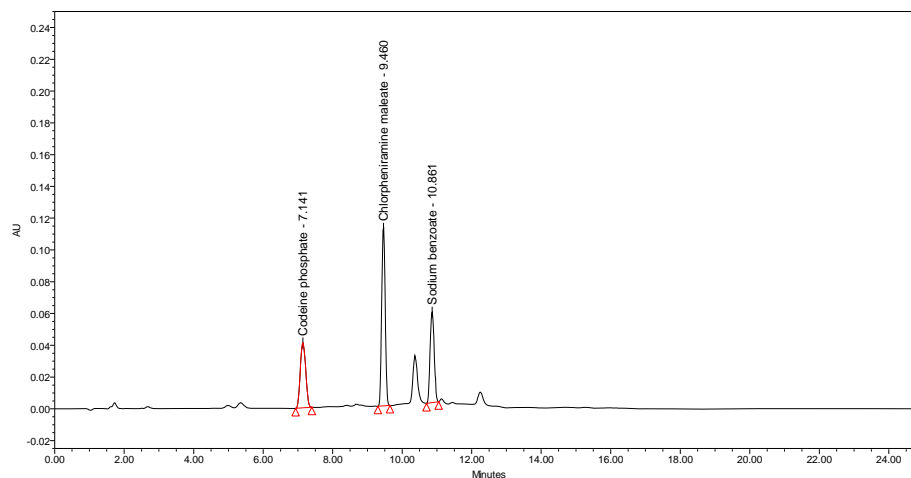
**Fig. No.29: Chromatogram showing Linearity level-2 (50%) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.149	294820		1.1	5266.45
Chlorpheniramine maleate	9.455	524063	10.1	1.0	21129.72
Sodium benzoate	10.858	306740	7.2	1.0	21087.85

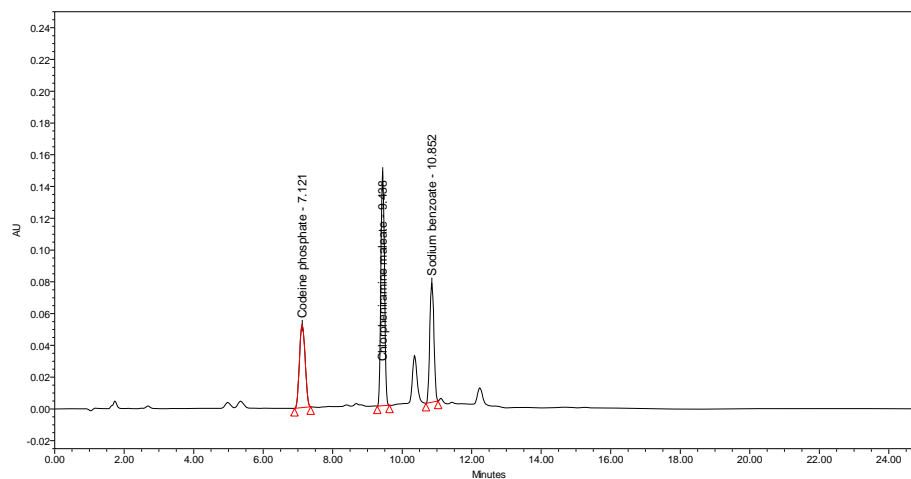


**Fig.No.30: Chromatogram showing Linearity level-3 (75%) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



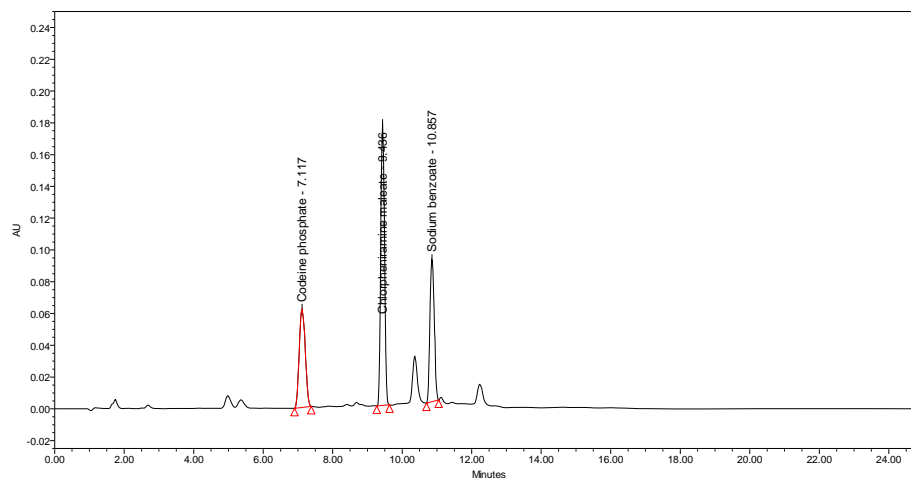
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.141	446999		1.1	3562.86
Chlorpheniramine maleate	9.460	792940	10.3	1.0	16129.42
Sodium benzoate	10.861	470036	7.1	1.1	17087.65

**Fig. No. 31: Chromatogram showing Linearity level-4 (100%) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



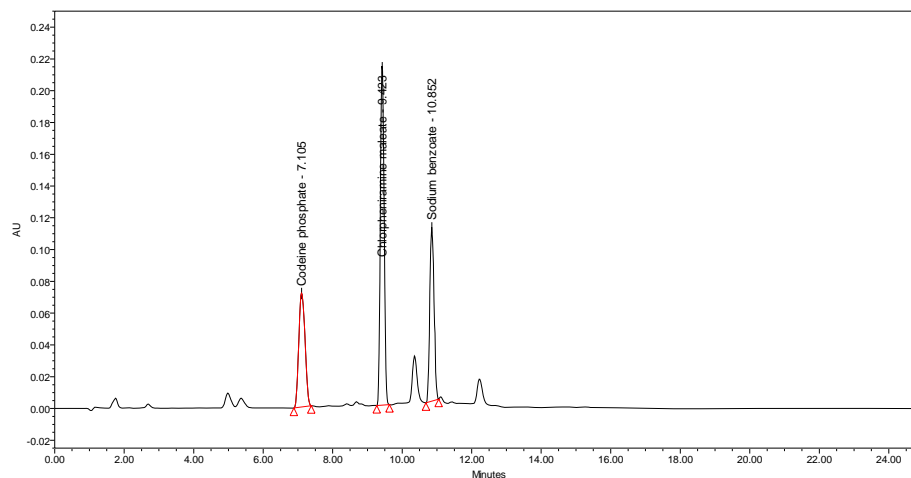
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.121	595115		1.1	12264.39
Chlorpheniramine maleate	9.438	1063971	10.4	1.0	48743.16
Sodium benzoate	10.852	626825	7.2	1.0	51955.02

**Fig. No. 32: Chromatogram showing Linearity level-5 (125%) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



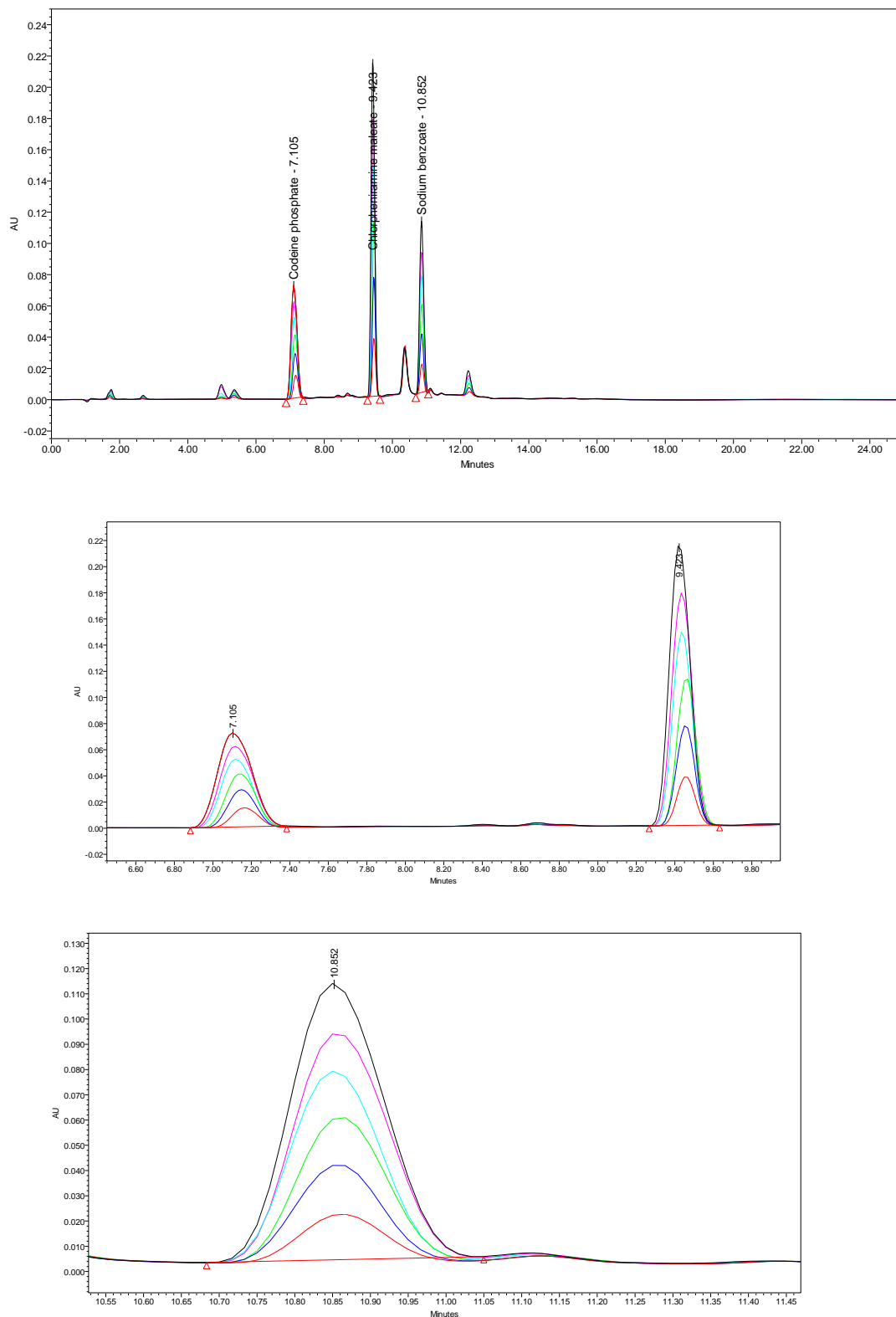
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.117	748334		1.1	14867.35
Chlorpheniramine maleate	9.435	1342834	10.5	1.0	50179.26
Sodium benzoate	10.857	779389	7.2	1.1	51087.86

**Fig. No. 33: Chromatogram showing Linearity level-6 (150%) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



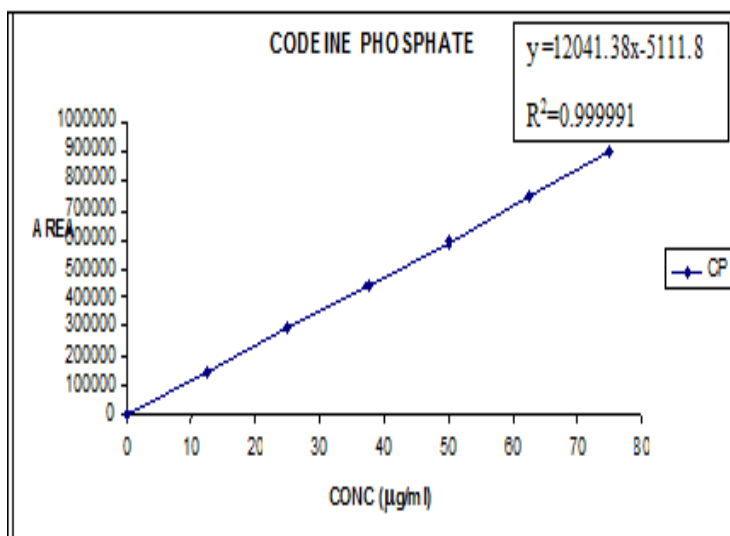
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.105	898406		1.1	176862.65
Chlorpheniramine maleate	9.423	1618163	10.1	1.0	521624.81
Sodium benzoate	10.852	936540	7.3	1.1	521087.45

**Fig. No. 34: Chromatogram showing overlay of Linearity levels of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



**Table No: 13 Table showing linearity result for Codeine phosphate.****For Codeine phosphate:**

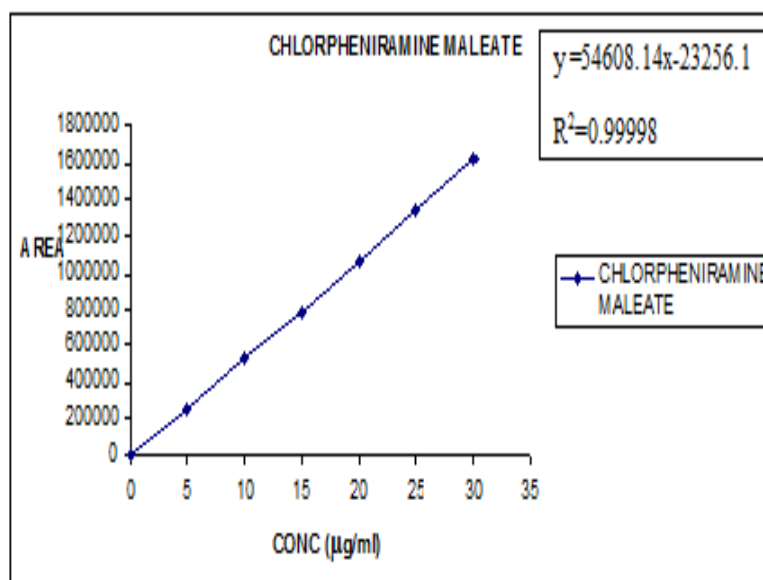
Linearity Level	Concentration (µg/mL)	Area
1	25	146517
2	50	294820
3	75	446999
4	100	595115
5	125	748334
6	150	898406
Correlation Coefficient		0.999991

**Fig.No.35: Figure showing Linearity graph of Codeine phosphate.**

The correlation coefficient value was found to be 0.999991.

**Table no: 14 Table showing linearity results for Chlorpheniramine maleate.****For Chlorpheniramine maleate:**

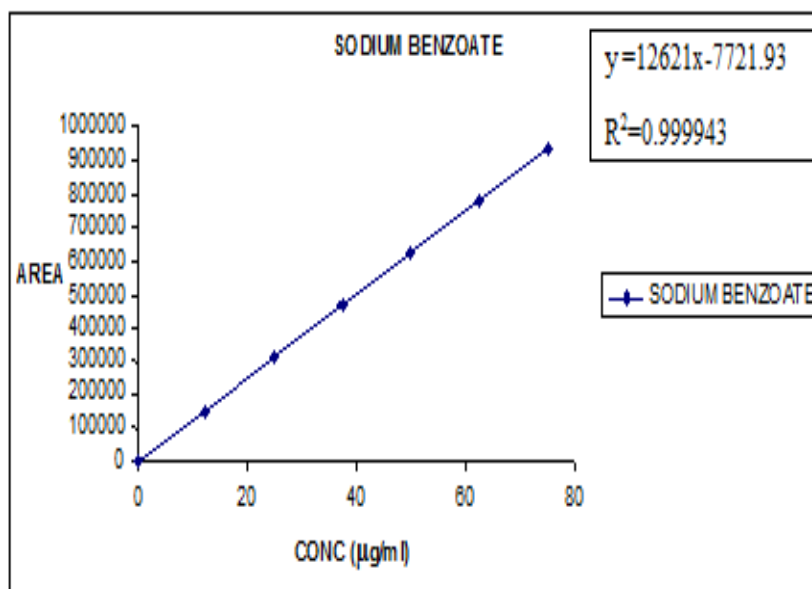
Linearity Level	Concentration (µg/ml)	Area
1	5	252347
2	10	524063
3	15	792940
4	20	1063971
5	25	1342834
6	30	1618163
Correlation Coefficient		0.999980

**Fig. No.36: Figure showing linearity graph of Chlorpheniramine maleate.**

The correlation coefficient value was found to be 0.999980.

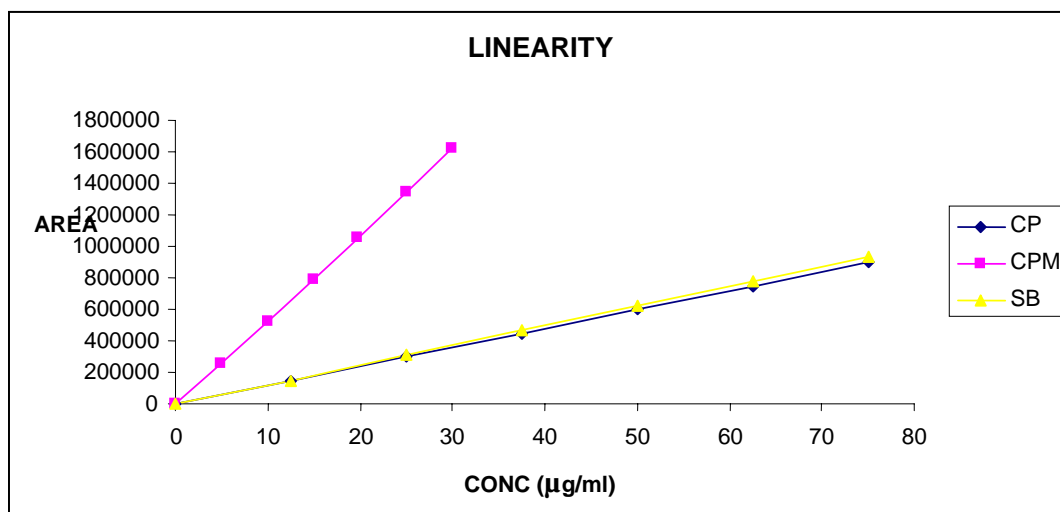
**Table no: 15 Table showing linearity results for Sodium benzoate.****For Sodium benzoate:**

Linearity Level	Concentration (µg/ml)	Area
1	12.5	147150
2	25	306740
3	37.5	470036
4	50	626825
5	62.5	779389
6	75	936540
Correlation Coefficient		0.999943

**Fig. No.37: Figure showing linearity graph of Sodium benzoate.**

The correlation coefficient value was found to be 0.999943.

**Fig.No.38: Figure showing Linearity graph of Codeine Phosphate, Chlorpheniramine Maleate and Sodium Benzoate in comparison.**



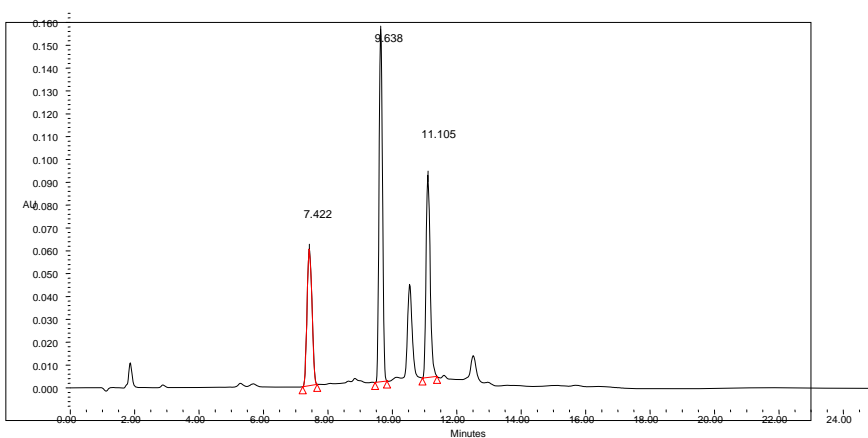
The correlation coefficient values were found to be within the acceptance limits for Codeine Phosphate, Chlorpheniramine Maleate and Sodium Benzoate.

#### **4.1.2.7 Robustness:**

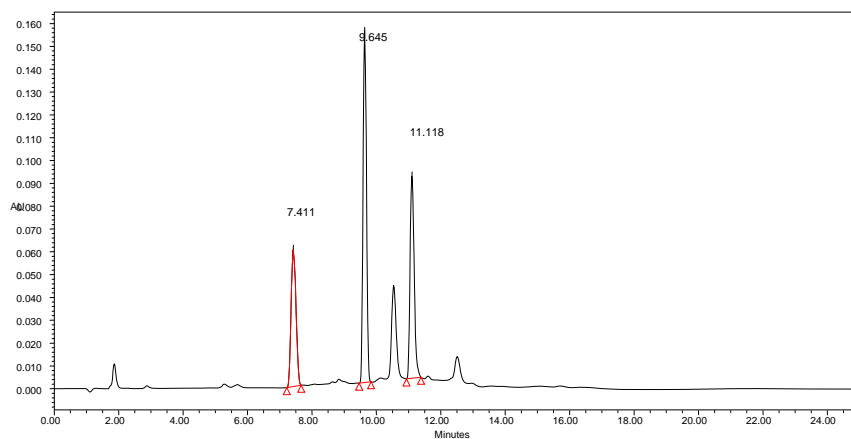
Robustness of the current method was investigated by analyzing samples of the drug product using the same chromatographic conditions set forth in method development but with a small change in the following chromatographic parameters:

- (a) Flow rate: 1.4 and 1.6 ml/min instead of 1.5 ml/min,
- (b) pH of the buffer preparation in mobile phase: 2.2 and 2.4 instead of 2.3.

%RSD of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate assay under these conditions is calculated and the results are shown in Table No: 16. The chromatograms are as shown in Fig. No: 39, 40.

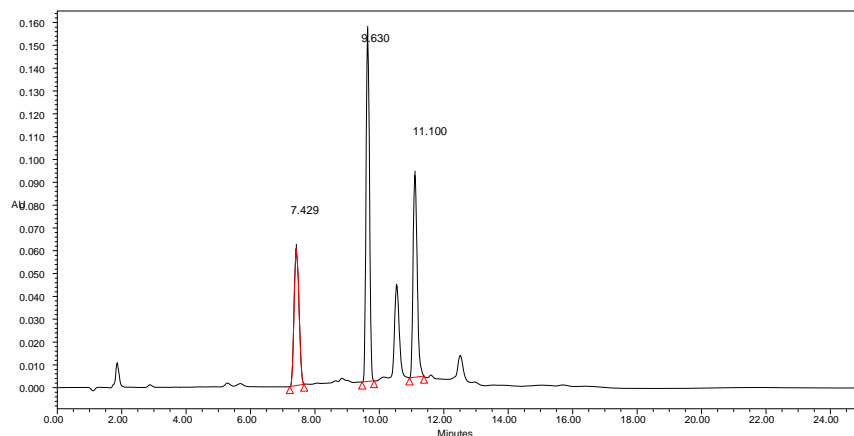
**A) Flow variation-1 (1.4mL/min):****Fig.No.39: Chromatogram showing Robustness-Flow variation (1.4ml/min) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.****Standard solution-1:**

Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.422	580770		1.1	16098.54
Chlorpheniramine maleate	9.638	1024792	11.9	1.0	58143.60
Sodium benzoate	11.105	723222	8.4	1.2	63871.82

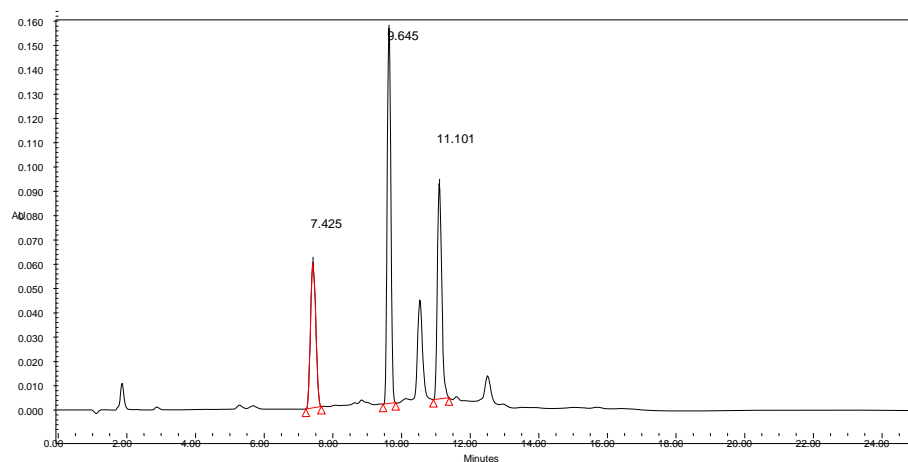
**Standard solution-2:**



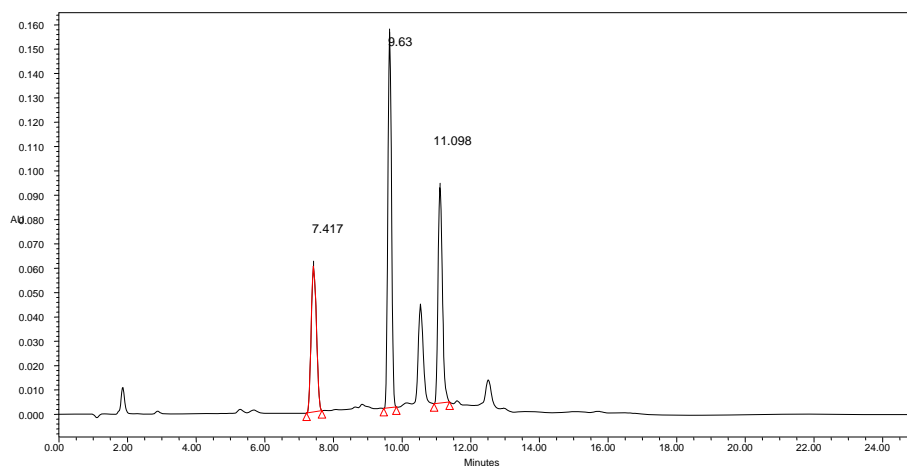
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.411	600775		1.0	16065.84
Chlorpheniramine maleate	9.645	1024795	11.8	1.0	58145.90
Sodium benzoate	11.118	723287	8.5	1.2	63878.12

**Standard solution-3:**

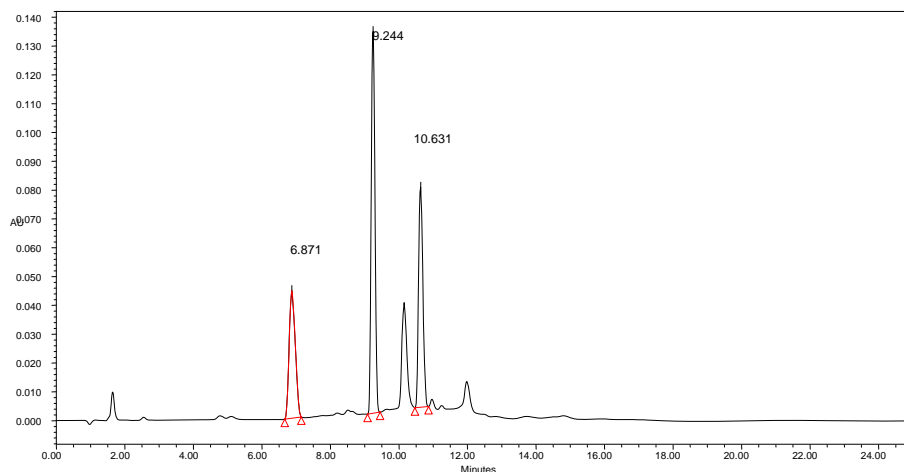
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.429	610720		1.0	16097.94
Chlorpheniramine maleate	9.630	11247986	11.9	1.0	58149.80
Sodium benzoate	11.100	723265	8.4	1.2	63876.72

**Standard solution-4:**

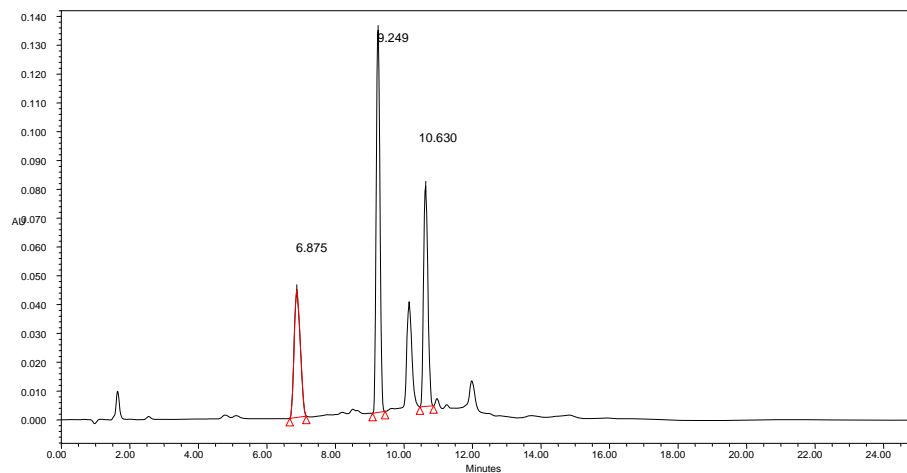
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.425	598670		1.0	16098.52
Chlorpheniramine maleate	9.645	1024892	11.8	1.0	57143.56
Sodium benzoate	11.101	723252	8.6	1.2	62871.85

**Standard solution-5:**

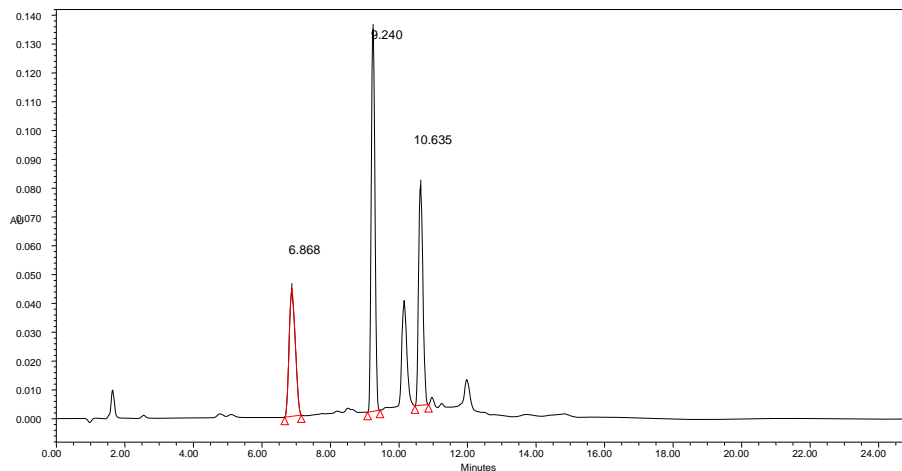
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.417	610770		1.0	15998.54
Chlorpheniramine maleate	9.31	1014792	12.1	1.0	59043.60
Sodium benzoate	11.098	713222	8.7	1.2	64271.82

**B) Flow variation-2 (1.6mL/min):****Fig. No. 40: Chromatogram showing Robustness-Flow variation (1.6ml/min) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.****Standard solution-1:**

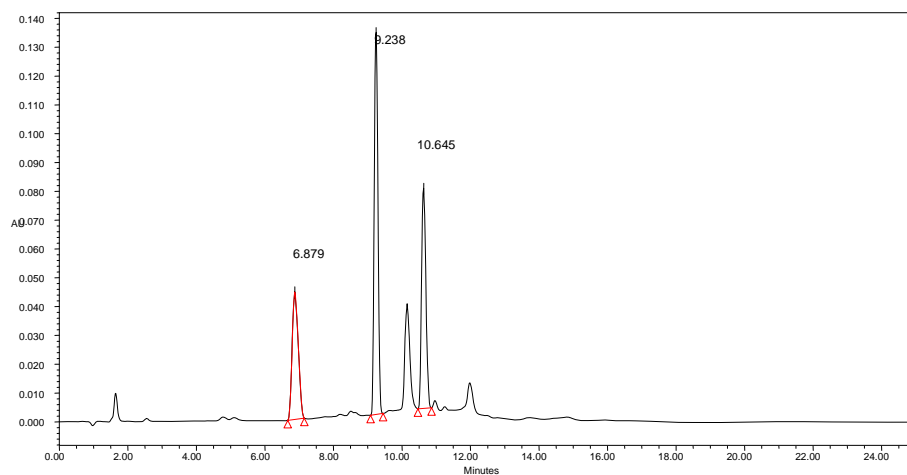
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	6.871	578798		1.1	16128.54
Chlorpheniramine maleate	9.244	1024769	11.8	1.0	58033.10
Sodium benzoate	10.631	723354	8.6	1.0	63872.42

**Standard solution-2:**

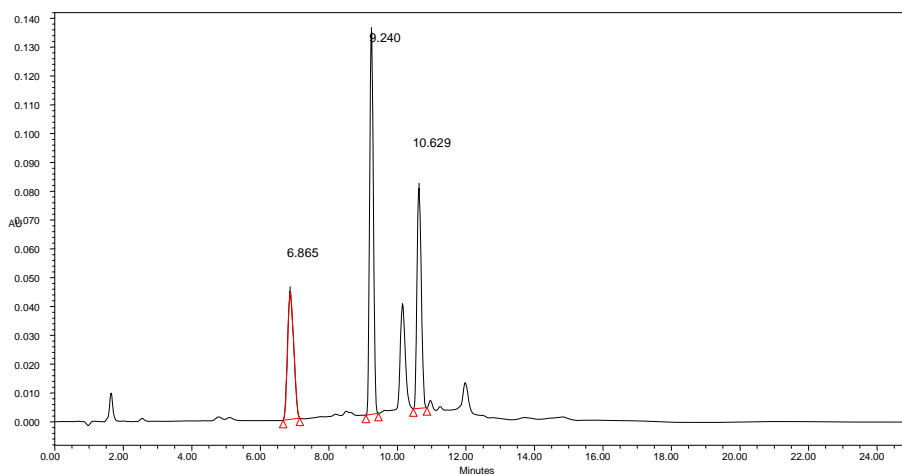
Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	6.875	578760		1.1	15998.94
Chlorpheniramine maleate	9.249	1024782	11.8	1.0	58146.86
Sodium benzoate	10.630	723232	8.2	1.0	63874.85

**Standard solution-3:**

Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	6.868	581765		1.1	16084.67
Chlorpheniramine maleate	9.240	1024789	11.7	1.0	58156.80
Sodium benzoate	10.635	723231	8.6	1.0	63869.92

**Standard solution-4:**

Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	6.879	580675		1.2	16076.84
Chlorpheniramine maleate	9.238	1024787	12.0	1.0	58155.90
Sodium benzoate	10.645	723232	8.8	1.1	63873.62

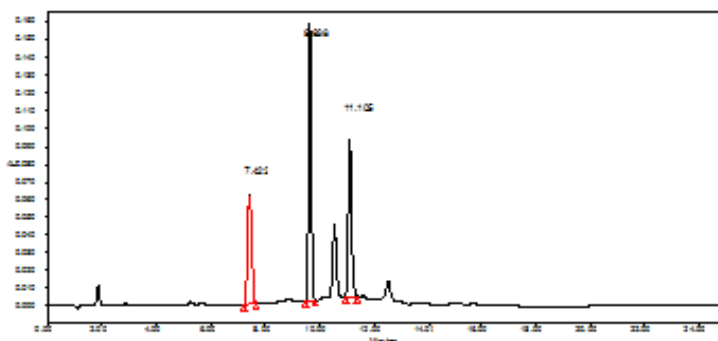
**Standard solution-5:**

Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	6.865	580762		1.1	16082.54
Chlorpheniramine maleate	9.240	1024785	11.7	1.0	58145.60
Sodium benzoate	10.629	723227	8.6	1.0	63876.82

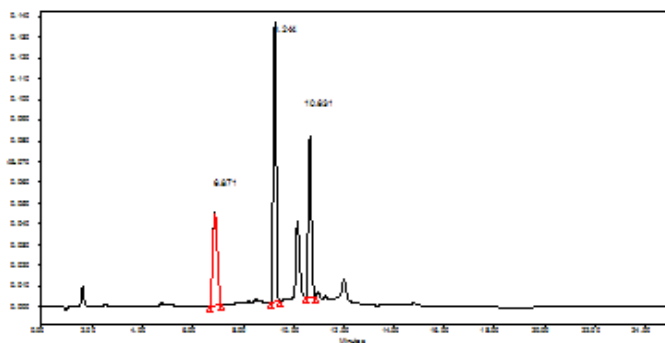
### C) P<sup>H</sup> variation-1 (2.2):

**Fig.No.41: Chromatogram showing Robustness- P<sup>H</sup> variation (2.2) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**

**Standard solution:**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.422	580770		1.1	16098.54
Chlorpheniramine maleate	9.638	1024792	11.9	1.0	58143.60
Sodium benzoate	11.105	723222	8.4	1.2	63871.82

**D) P<sup>H</sup> variation-2 (2.4):****Fig.No.42: Chromatogram showing Robustness- P<sup>H</sup> variation (2.4) of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.****Standard solution:**

Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	6.871	578798		1.1	16128.54
Chlorpheniramine maleate	9.244	1024769	11.8	1.0	58033.10
Sodium benzoate	10.631	723354	8.6	1.0	63872.42

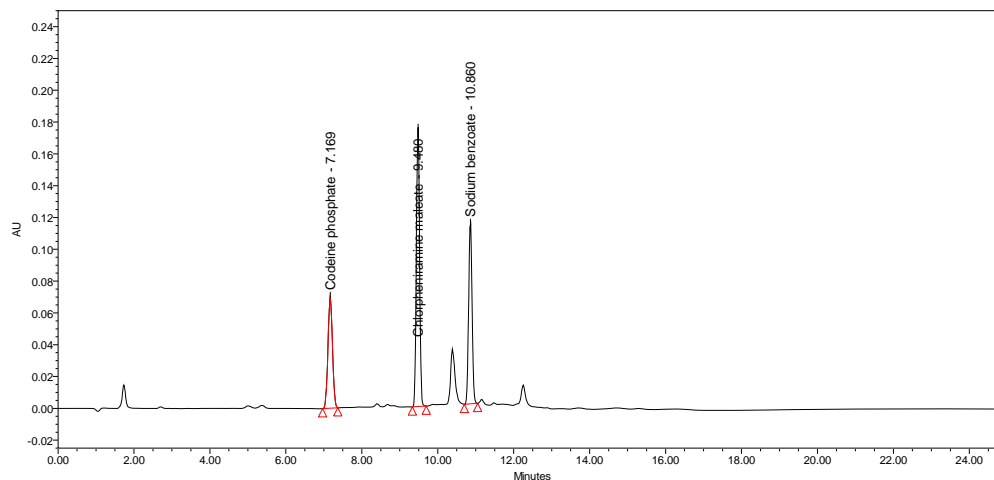
**Table No: 16 Table showing Robustness results for change in flow rate and pH of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**

Flow rate	Inj.sample	Plate count	Tailing	RT	%RSD
1.4ml/min	Codeine phosphate	16098.54	1.0	7.148	0.42
	Chlorpheniramine maleate	58143.60	1.04	9.469	0.36
	Sodium benzoate	63871.82	1.1	10.86	0.38
1.6ml/min	Codeine phosphate	13652.01	1.23	7.105	0.40
	Chlorpheniramine maleate	51711.86	1.0	9.420	0.35
	Sodium benzoate	57803.38	1.0	10.85	0.37
<b>pH variation</b>					
2.2	Codeine phosphate	17779.21	1.2	7.180	0.41
	Chlorpheniramine maleate	52624.93	1.2	9.394	0.37
	Sodium benzoate	71472.43	1.1	10.923	0.39
2.4	Codeine phosphate	17768.98	1.1	7.165	0.45
	Chlorpheniramine maleate	52628.56	1.0	9.35	0.33
	Sodium benzoate	71470.75	1.0	10.910	0.40

#### 4.1.2.8 Ruggedness:

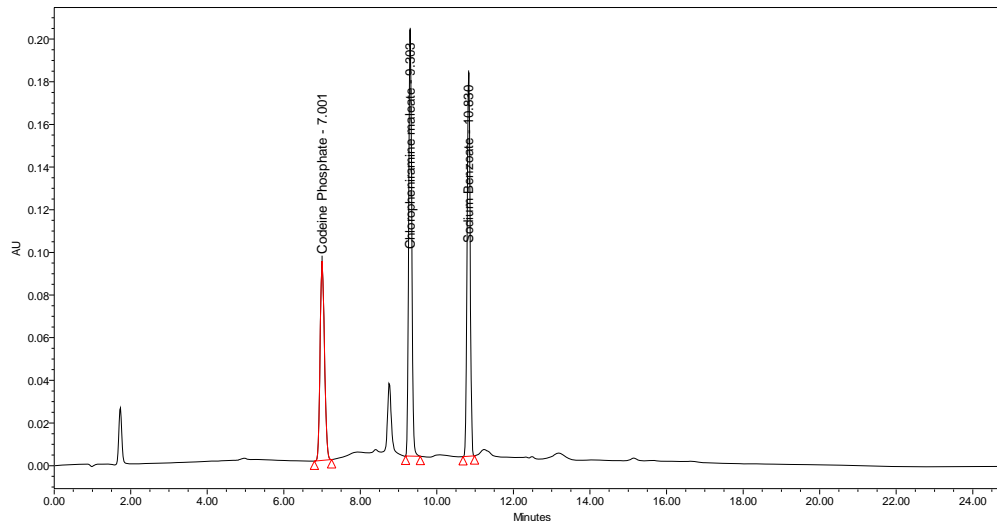
Ruggedness of the current method was demonstrated by analyzing three samples (assay) of syrup formulation by two analysts in the same laboratory on two different days. The %RSD values for the 12 samples are calculated to be 0.42%, 0.62%, and 0.49% for codeine phosphate, chlorpheniramine maleate and sodium benzoate respectively. The chromatograms are as shown in Fig.No:41, 42 and the results are as shown in the Table No: 17 indicating the ruggedness of the method.

**Fig. No. 43: Chromatogram showing Ruggedness: Day-1, Analyst-1 of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.169	566151		1.0	13652.01
Chlorpheniramine maleate	9.480	1023254	11.2	1.0	51711.86
Sodium benzoate	10.860	699694	8.0	1.0	57803.38

**Fig. No.44: Chromatogram showing Ruggedness: Day-2, Analyst-2 of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**



Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Codeine phosphate	7.001	566151		1.0	13658.61
Chlorpheniramine maleate	9.283	1023254	10.8	1.1	51715.96
Sodium benzoate	10.860	699694	7.8	1.0	57823.28



**Table No: 17 Table showing Ruggedness results of Analysts-1&2 on Days-1&2 of Codeine phosphate, Chlorpheniramine maleate and Sodium Benzoate.**

	<b>ANALYST-1</b>					
	<b>DAY-1</b>			<b>DAY-2</b>		
<b>A.I</b>	<b>CP</b>	<b>CPM</b>	<b>SB</b>	<b>CP</b>	<b>CPM</b>	<b>SB</b>
<b>1.</b>	593892	1024792	727778	584035	1047999	725842
<b>2.</b>	593436	1023254	735642	592456	1036824	724689
<b>3.</b>	593945	1025256	731028	586945	1047642	723688
<b>Mean</b>	593757	1024434	731482	587812	1044155	734739
<b>SD</b>	2796.98	1047.9	3159.6	4276.9	6351.3	1077.8
<b>%RSD</b>	0.40	0.103	0.54	0.72	0.61	0.15

	<b>ANALYST-2</b>					
	<b>DAY-1</b>			<b>DAY-2</b>		
<b>A.I</b>	<b>CP</b>	<b>CPM</b>	<b>SB</b>	<b>CP</b>	<b>CPM</b>	<b>SB</b>
<b>1.</b>	590077	1053039	723222	591486	1052786	725846
<b>2.</b>	584628	1048958	724674	587628	1058462	725486
<b>3.</b>	587463	1035478	723654	584968	1059466	735644
<b>Mean</b>	587389	1045825	723850	588027	1056904	728992
<b>SD</b>	2725.2	9190.15	745.57	3277.3	3602.02	5763.6
<b>%RSD</b>	0.46	0.87	0.103	0.55	0.34	0.79

## 4.2 SUMMARY AND CONCLUSION

A new method of analysis is developed for identification and quantification of Codeine phosphate, Chlorpheniramine maleate drugs and Sodium benzoate preservative by RP-HPLC method. The sample preparation is simple and the analysis time is short. The analytical procedure is validated as per ICH Q2B guidelines and shown to be accurate, precise and specific.

This method represents a fast analytical procedure for the simultaneous quantitation of Codeine phosphate, Chlorpheniramine maleate and Sodium benzoate. The method is amenable to the routine analysis of large numbers of samples with good precision and accuracy.

---

**5. BIBLIOGRAPHY**

1. Sethi.PD. HPLC Quantitative analysis of pharmaceutical formulations, Ed-1<sup>st</sup>, CBS Publishers and distributors, New Delhi, 2001, pp: 94-105.
2. Glajch L, Kirkaland J and Snyder R. Practical HPLC method development, Ed-2<sup>nd</sup>, John Wiley and sons, INC, New York, 1999, pp: 234-260.
3. Ashutoshkar S. Pharmaceutical Drug Analysis, Ed-2<sup>nd</sup>, New age international (p) limited publishers, 2005, pp: 452-474.
4. <http://en.wikipedia.org/wiki/Chromatography>.
5. U.S.P. Asian Edition, United Pharmacopoeial Convention Inc, Rockville, 2005, pp: 2386-2389.
6. James.W Munson. Modern Methods of Pharmaceutical Analysis, Medical Book Distributors, Mumbai, 2001, pp: 17-54.
7. Sethi P.D. High Performance Liquid Chromatography and Quantitative analysis Pharmaceutical Formulations, Ed-1<sup>st</sup>, CBS Publishers and Distributors, 2001, pp: 3-5.
8. Erwing, G. W. Instrumental Methods of Chemical Analysis, Ed-2<sup>nd</sup>, McGraw Hill Book Company, 1960, pp: 3-5.
9. GurdeepR.Chatwal and Sham K.Anand. Instrumental Methods of Chemical Analysis, Ed-5<sup>th</sup>, Himalaya Publishing House, pp: 1.2 – 1.5.
10. Sharma B.K. Instrumental Method of Chemical Analysis, Ed-23<sup>rd</sup>, Goal publication House, 1991, Merrut, pp: 7-10.
11. RamaRao, G.Murthy, S.S.N and Khadgapathi P. High Performance Liquid Chromatography and its role in pharmaceutical analysis, Eastern Pharmacist, 1986, 20 (346), pp: 53-59.

12. Sastry C.S.P., Prasad T.N.V. and Rao E.V. Recent application of High Performance Liquid Chromatography in Pharmaceutical Analysis, Indian. Pharm. Education, 1987, 21, pp: 37-40.
13. Loyd. K. Snyder, Joseph. J. Kirkland and Joseph. L. Glajch. Practical HPLC Method Development, Ed-2<sup>nd</sup>, Wiley – Interscience Publication, USA, pp: 742-747.
14. Willard, H.Y. Merritt L.L., Dean J.A. and Settle F.A. Instrumental Methods of Analysis, Ed-7<sup>th</sup>, CBS Publishers and Distributors, New Delhi, 1991, pp: 436-439.
15. The International Conference on Harmonization (ICH)-Validation of Analytical Procedures: Methodology (Q2B), Food and Drug Administration, USA, Nov. 1996.
16. [http://en.wikipedia.org/wiki/Codeine\\_phosphate](http://en.wikipedia.org/wiki/Codeine_phosphate).
17. [http://en.wikipedia.org/wiki/Chlorpheniramine\\_maleate](http://en.wikipedia.org/wiki/Chlorpheniramine_maleate).
18. [http://en.wikipedia.org/wiki/Sodium\\_benzoate](http://en.wikipedia.org/wiki/Sodium_benzoate).
19. Fuad Al-Rimawi. Normal-phase LC method for simultaneous analysis of pseudophedrine hydrochloride, dextromethorphan hydrobromide, chlorpheniramine maleate and paracetamol in tablet formulations. *Saudi Pharmaceutical Journal*. 2010; 18(2): 103-106.
20. Mukesh Maithani, Richa Raturi, Vertika Gautam, Dharmendra Kumar, Amrendra Kumar Chaudhary, Anand Gaurav and Ranjit Singh. Development And Validation Of A Rp-Hplc Method For The Determination Of Chlorpheniramine Maleate And Phenylephrine In Pharmaceutical Dosage Form. *International Journal of Comprehensive Pharmacy*. 2010; 01(05):1-4.

21. Adnan Manassra , Mustafa Khamis, Magdy el-Dakiky, Zuhair Abdel-Qader and Fuad Al-Rimawi. Simultaneous HPLC analysis of pseudophedrine hydrochloride, codeine phosphate and triprolidine hydrochloride in liquid dosage forms. *Journal of Pharmaceutical and Biomedical Analysis*.2010; 51(4):991-993.
22. Ezzat M. Abdel-Moety. Selective Hplc-quantification of promethazine Hydrochloride and its degradation products In presence of sodium benzoate. *Saudi Pharmaceutical Journal*. 2009; 17(2):161-169.
23. Aravindaraj Joghee Rajua. Development and Application of a Validated Liquid Chromatography-Mass Spectrometry Method for the Determination of Dexchlorpheniramine Maleate in Human Plasma. *Iranian Journal of Pharmaceutical Sciences*.2007; 3(3):161-170.
24. Maria-Elisa Capella-Peiro. Optimization of a capillary zone electrophoresis method by using a central composite factorial design for the determination of codeine and paracetamol in pharmaceuticals. *Journal of Chromatography B*.2006; 839(1-2):95-101.
25. Maria R. Gomez. Development and validation of a capillary electrophoresis method for the determination of codeine, diphenhydramine, ephedrine and noscapine in pharmaceuticals. *Il Farmaco*.2005; 60(1):85-90.
26. V.Galli. High performance liquid chromatographic analysis of dextromethorphan, guaifenesin and benzoate in a cough syrup for stability testing. *Journal of Chromatography A*.2004; 108(2):207-211.
27. Mohsen Kompany-Zareh. Spectrophotometric resolution of ternary mixtures of pseudoephedrine hydrochloride, dextromethorphan hydrobromide, and sodium benzoate in syrups using wavelength selection by net analyte signals

- calculated with hybrid linear analysis. *Analytica Chimica Acta*.2004; 526(1):83-94.
28. A.Garcia. Poly(ethyleneglycol) column for the determination of acetaminophen, phenylephrine and chlorpheniramine in pharmaceutical formulations. *Journal of Chromatography B*.2003; 785(2):237-243.
29. Darryl J. Hood. A chromatographic method for rapid and simultaneous analysis of codeine phosphate, ephedrine HCl and chlorpheniramine maleate in cough-cold syrup formulation. *Journal of Pharmaceutical and Biomedical Analysis*.2003; 30(5):1595-1601.
30. A.Marin. Validation of a HPLC quantification of acetaminophen, phenylephrine and chlorpheniramine in pharmaceutical formulations: capsules and sachets. *Journal of Pharmaceutical and Biomedical Analysis*.2002; 29(4): 701-714.
31. Murat Kartal. LC method for the analysis of paracetamol,caffeine and codeine phosphate in pharmaceutical preparations. *Journal of Pharmaceutical and Biomedical Analysis*.2001; 26(5-6):857-864.
32. Ruby Ragonese. Full and fractionated experimental designs for robustness testing in the high-performance liquid chromatographic analysis of codeine phosphate, pseudoephedrine hydrochloride and chlorpheniramine maleate in a pharmaceutical preparation. *Journal of Chromatography A*.2000; 870(1-2):45-51.
33. Io-Wah Lau. High-performance liquid chromatographic determination of active ingredients in cough-cold syrups with indirect conductometric detection. *Journal of Chromatography A*.1995; 693(17):45-54.

34. Brianne Weingarten. Determination of codeine in human plasma by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A*.1995; 696(1):83-92.
35. G. Santoni. Simultaneous determination of aspirin, codeine phosphate and propyphenazone in tablets by reversed-phase high-performance liquid chromatography. *International Journal of Pharmaceutics*.1992; 80(1-3):263-266.
36. Zhao Rong Chen. Simultaneous determination of codeine, norcodeine and morphine in biological fluids by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography B*.1989; 491:367-378.
37. O. W. Lau. The simultaneous determination of active ingredients in cough-cold mixtures by isocratic reversed-phase ion-pair high-performance liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis*.1989; 7(6):725-736.
38. Ron Ginman. Simultaneous determination of codeine and ibuprofen in plasma by high-performance liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis*.1985; 3(5):439-445.